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THE MORPHOLOGY OF SOME PROTOZOAN PARASITES IN RELATION TO MICROTECHNIQUE*

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INTRODUCTION

The Importance of Technique

To a considerable extent the diagnosis of parasitic protozoa, and certainly the study of their detailed morphology, is dependent upon the application of technical processes of fixation and staining, and the necessary accessory procedures. It might be well to emphasize that in the experience of my co-workers and myself, the diagnosis of intestinal protozoa is very much aided by the study of properly prepared fixed and stained smears. In our survey of college students (Wenrich, Stabler, and Arnett, 1935), we made a comparison of the relative efficiency of the examination of fresh smears in saline and iodine with that of the examination of fixed and stained slides. For 700 of the students, the stained slides furnished 11.2 per cent more positives than the fresh preparations. The advantages were different for different species. For example, 15.2 per cent more positives for *Endolimax nana*, 34.5 per cent more for *E. histolytica*, and 61.3 per cent more for *Dientamoeba* were detected through the study of stained slides alone than were seen on the fresh preparations alone. The tendency to get away from the study of prepared slides in making such surveys is to be deplored.

Because of the importance of the utilization of appropriate techniques in making permanent preparations, it seems worth while to consider some aspects of microtechnique in relation to the morphology of these parasites. In the following presentation consideration will be given primarily to intestinal protozoa.

For more than twenty years I have given much attention to intestinal protozoa and have therefore been forced to evaluate the relation between their apparent organization and the techniques employed in their preparation for study. It was soon realized that many protozoa present different appearances when treated in different ways and a certain amount of ex-

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perimentation with techniques was a natural result. Among the other conclusions from this study may be noted (1) that the same technique does not always produce the same result with the same species of parasite, because of the variability of the parasites themselves and the variability of the environments in which they are found; and (2) that different species often give very different results to the same technique because of inherent specific differences.

Results Previously Published

In successive publications from my laboratory, dealing with intestinal protozoa, some results of the employment of various techniques have been reported. I may refer to the work on *Trichomonas muris* (1921) in which it was noted that Schaudinn's fluid produced more shrinkage than Allen's modification of Bouin's; that cytoplasmic vacuoles showed stainable contents after fixation with sublimate-acetic but did not after fixation with Schaudinn's fluid; also that the parabasal body of this flagellate did not usually stain with Heidenhain's hematoxylin after fixation with Schaudinn's but did stain after Flemming's fluid and others containing chromic acid or osmic acid or both. Further, reference may be made to the studies of Freeman (1929) and Stabler (1932b) showing that various reagents were effective in producing "buds" on the cysts of *Entamoeba coli*, and that of Segal (1932) who reported similar results for *Endolimax nana*. In 1932 Stabler showed that when 15 to 20 per cent of acetic acid was added to the Schaudinn's fluid in which *Endolimax nana* was fixed, the peripheral layer of the nucleus in this species stained more distinctly with iron hematoxylin, while the endosome stained more faintly. In a joint note with Doctor Geiman (1933) it was pointed out one-half-strength Schaudinn's fluid gave as satisfactory a fixation of many intestinal protozoa as the full-strength mixture. The study on *Iodamoeba* (1937b) showed that higher concentrations of acetic acid in Schaudinn's fluid produced less stainability of the endosome of the cyst nuclei and that hemalum did not stain the endosome of cyst nuclei as intensely as Heidenhain's hematoxylin. In a paper on *Dientamoeba* (1937a) it was shown that the nuclei of this species usually fail to stain after fixation with Schaudinn's fluid unless a relatively high percentage of acetic acid is added, and that excellent stainability usually follows fixation with Bouin's or Hollande's fluids. The paper on nuclear structure and division in *Entamoeba muris* (1940) recorded among other things that the endosome does not stain with hemalum after fixation with Schaudinn's fluid and that there are two groups of chromosome-like bodies in the dividing nucleus one of which reacts to the Feulgen technique and the other does not, and finally reference may be made to the recent progress reports in the Yearbook of the American Philosophical

Society in which some of the results of more systematized experimentation techniques are briefly recorded (1938).

It will be both impossible and inappropriate to record all the variations in technique that have been followed during the past twenty years. Several thousand slides have been made and examined and over 130 different chemical agents or dilutions or combinations of them have been tried as fixatives. Not so much has been attempted with different staining methods. Certain of the more important results will be presented.

Acknowledgments

Since the material on which these studies have been based has been accumulated over a period of more than 20 years, I am indebted to many sources and a good many individuals for assistance. For financial assistance I am indebted to the Special Research Fund of the University of Pennsylvania, the Penrose Fund of the American Philosophical Society and a special research fund contributed by John Wyeth and Brother of Philadelphia. For assistance at various times in carrying out technical processes I am indebted to Doctor Quentin M. Geiman, Doctor R. M. Stabler, and especially to Doctor Sarah H. Stabler. For assistance in securing source material I am indebted to a good many persons but more especially to Doctors A. D. Waltz, R. M. Stabler, John H. Arnett, H. A. Shelanski, J. H. Clark, A. L. Luchi, and M. M. Rothman, and Mr. R. L. Brown. Mr. Brown also made the drawings for Figs. 71 and 72.

COMMENTS ON TECHNICAL PROCESSES

Routine Procedures

Fecal smears were made by diluting feces with modified Ringer's solution to make a thin paste which was spread out on a cover glass with the curved points of a small pair of forceps. The smear was then dropped smear-side down on the surface of the fixing fluid which was usually placed in a Petri dish. The smear was then turned over and placed with the smear-side up on the bottom of the dish. The first washing was generally in 50 per cent alcohol, after which the preparations were transferred to 70 per cent alcohol where they were kept until staining was undertaken. The graded alcohol series was usually 10, 30, 50, 70, 83, and 95 per cent, or the lowest three were 5, 15, and 30 per cent. From 95 per cent alcohol the preparations were cleared in a mixture of oil of thyme (3 or 4 parts) and oil of cloves (1 part) followed by xylol.

The Feulgen technique used was the standard one described in McClung's (1937) Handbook of Microscopical Technique, usually accompanied with Fast Green as a counter stain. In using iron hematoxylin, 4 per cent iron alum was used as the mordant, customarily for 24 hours or overnight, and 0.5 per cent aqueous hematoxylin as the stain, for an equal length of time.

Temperature

It is generally agreed that a warm reagent will act more rapidly than a cold one. It is a common practice to warm fixing solutions before use. Some years ago Miss Miriam Scott (now Mrs. A. M. Lucas) conducted temperature experiments for me with Schaudinn's fluid as the fixative and *Paramecium* as the experimental material. She found that fixing at 45 degrees C gave the most satisfactory results. Since that time I have fixed material at between 40 and 50 degrees or at room temperature. With the common intestinal protozoa, I have been unable to detect any significant difference as between fixation at the room or higher temperatures; hence, nearly all experimental fixations have been made at room temperature.

Fixation Time

It is customary to fix blocks of tissue for hours or days in many of the common fixing agents. It would seem obvious that thin smears would not require so much time. My experiments have shown that, in general, fixation for one minute produces as satisfactory a result as fixation for one to many hours. Often the shorter exposures produce better results than the longer ones.

Heidenhain's Hematoxylin

Iron alum hematoxylin is widely used and has the great merit of giving sharp differentiations of black-stained parts against backgrounds containing little or no stain. It is commonly employed for sectioned material and for smears. There are some obvious disadvantages in its use for smears. A relatively slight difference in thickness of a smear may make the difference between proper differentiation in the thinner areas and undestained black in the thicker areas, or, if the thicker areas are destained enough, the thinner ones become unstained. Bulky protozoa, such as many of the ciliates, do not usually give good results because of their too-great thickness. Some of the difficulties can be overcome by destaining with very weak iron alum, such as 1.0 or even 0.5 per cent, as recommended by Sharp (1914), or by destaining with a saturated aqueous solution of picric acid as suggested by Tuan (1930). Alcoholic solutions of picric acid, e.g., in 50 or 70 per cent, will destain much more rapidly, but the more rapid action may nullify the benefits of the slower action of the aqueous solution. I have found the aqueous picric destaining method advantageous for amoebae, ciliates and certain sporozoa. It has not been so successful for flagellates, because the differentiation of cytoplasmic structures is not so sharp as when destaining is done with 2 per cent iron alum.

The iron hematoxylin method as described in the books calls for mordanting for from 2 to 24 hours and staining for an equal length of time.

When quick results are not required it is often convenient to employ the 24-hour schedule. If haste is essential, the time can be reduced to 20 to 30 minutes at room temperature or even to 2 to 5 minutes if the temperature is raised to 35 or 40 degrees C as suggested by Craig and Faust (1937). I find these short-time treatments to be serviceable for diagnostic purposes, but they are often more capricious and less enduring than the results of the longer treatments.

Other Stains

I have experimented relatively little with different stains. The long time usually allowed for Heidenhain's hematoxylin makes it desirable to find a less time-consuming substitute for diagnostic purposes, at least. In many laboratories, Delafield's hematoxylin is the standard stain for tissue sections, and it has naturally been employed also for smears of intestinal protozoa. In my experience this stain is not very satisfactory for routine practice. Hemalum is quicker and, I believe, gives better results. It is not supposed to overstain, but if it does, it may be slowly destained with one per cent solution of potassium alum. After staining and washing the smear should be thoroughly alkalized either by a longer washing in tap water or a treatment with water containing a trace of ammonia. This stain is not advised for flagellates, since it does not stain cytoplasmic structures well. It may also fail to stain the endosomes of endozoic amoebae, hence its use must be accompanied by proper precautions in interpretation. Phosphotungstic acid hematoxylin has been recommended by some technicians, but it is less satisfactory than Heidenhain's. It may stain elements not stained by Heidenhain's and hence is valuable as a member of a series designed to bring out all possible structures.

Feulgen's Nucleal Reaction

It has often been surprising that a standard Feulgen procedure which results in typical coloring of nuclear chromatin in metazoan nuclei, may show a very weak coloring or none at all in the nuclei of protozoa. Nuclei of one stage in the life history of a protozoan may show a reaction while those of another stage do not. These diversities merit some comment.

Morris (1935) and Sassuchin (1935) reported that the nucleus of *Endamoeba blattae* showed no coloring by the Feulgen technique. Meglitsch (1939), however, found that while the nuclei of this amoeba failed to react in the non-dividing stage, when nuclear division took place, the chromosomes gave a positive reaction. In my own slides made from the intestines of cockroaches and treated by the Feulgen method, there were so few individuals of *E. blattae* that I could not determine the reaction for this species. However, the goodly number of specimens of *E. thomsoni* all showed the central granules of the nucleus giving a definite although not very strong Feulgen reaction.

For gregarines I obtained results similar to those reported by Reichenow (1928). In smears from the seminal vesicles of earthworms containing many acephaline gregarines fixed in alcoholic Bouin's fluid, and treated by the Feulgen method, the nuclei of the trophozoites failed to show a reaction although those of the spores and the dividing nuclei in the cysts all showed the typical coloring. On slides of *Cryptobia* (*Trypanoplasma*) from fresh water fish, the Feulgen technique produced a faint reaction in the nucleus but the kinetoplast (parabasal) was deeply colored. Jirovec (1927), Reichenow (1928), Cunha and Muniz (1928) and Sas-suchin (1935) have reported similar results with hemoflagellates. Margolena (1932) and others have called attention to the fact that plant materials containing aldehydes, such as lignin, suberin, etc., give a positive nucleal reaction and on fecal smears it is common to see such materials showing the Feulgen coloration. It may therefore be stated (1) that nuclear chromatin does not always give a Feulgen reaction, and (2) that not everything that colors with Feulgen is chromatin. One might even question the frequently made statement that the Feulgen reaction is specific for thymo-nucleic acid.

For staining reactions in general, the dispersion factor is important. If a stainable material is finely divided and scattered among non-staining materials, the stain, if any, in these particles may be overlooked or masked by the associated substances. In the trophozoites of gregarines, for example, the failure of the nuclei to show a positive Feulgen coloration may be due to the great dispersion of the chromosome-forming materials in the relatively large nucleus which contains a large amount of non-chromosome-forming "chromatin." As a matter of fact, a close examination of some of these nuclei reveals the presence of very small scattered granules that show a faint Feulgen coloration. When this material is condensed into chromosomes at the time of nuclear division, the coloring is much more readily recognized.

In spite of the somewhat indefinite results obtained by the Feulgen method, it is, nevertheless, an important technical procedure and has proven to be very valuable in the interpretation of nuclear substances. Interpretation here, as always, needs to be made with due caution.

Fixing Agents

As soon as one attempts to experiment with different fixing agents one becomes aware that different solutions give different results. Here some comments will be made upon the use of some of the fixing agents used for fixing intestinal protozoa.

Schaudinn's sublimate alcohol, especially when a small amount of acetic acid is added, is probably the most generally useful fixing solution for intestinal protozoa. As a rule it provides for excellent nuclear dif-

ferentiation partially because it renders many cytoplasmic elements unstainable with the commonly employed stains. Fibrillar structures and basal bodies of flagellates and ciliates usually stain well but chondriosomes and some other cytoplasmic inclusions do not. This fixing agent usually produces considerable shrinkage, and for larger protozoa, like the large ciliates and gregarines, the distortion is often excessive. For these larger species, other fixing agents often give better results, such as Perenyi's fluid, Kleinenberg's fluid and Petrunkevitch's fluid for ciliates and alcoholic Bouin's for gregarines.

Bouin's fluid, especially the modification with saturated picric acid 75 parts, formol 15 parts and glacial acetic 10 parts, does tolerably well for the trophic stages of intestinal protozoa but it does not penetrate cysts well. It is excellent for *Dientamoeba*. Other standard fixing solutions, such as Hollande's, picromercuric, picroacetic, Kleinenberg's, Gilson's, Carnoy's, Destin's, Worcester's, and Flemming's fluids all have their usefulness for special purposes but do not possess the general adequacy that Schaudinn's fluid does.

The one-half strength Schaudinn's fluid recommended by Doctor Geiman and myself (1933) has proven to be very satisfactory for practically all the intestinal protozoa on which it has been tried. Even one-fourth strength has proven to be a satisfactory fixing agent for *Endolimax*, *Iodamoeba*, and *Entamoeba histolytica*. One-half strength Bouin's fluid has also proven to be as good as full strength when tested on *Entamoeba histolytica* and *Endolimax nana*.

When a saturated solution of mercuric chloride was diluted to three-fourths, two-thirds, one-half, one-third, one-fourth, and one-tenth strengths, the weaker solutions, that is, one-half or less, proved to be more satisfactory fixing agents than strong concentrations. Fifty per cent alcohol causes much less distortion of cytoplasm than stronger concentrations, although nuclear staining is much the same in all. Low concentrations of acetic acid, such as one, two, and five per cent proved to be more satisfactory fixing agents than stronger solutions. With picric acid, however, 50 per cent or higher percentages of a saturated solution gave better results than lower concentrations. One per cent hydrochloric acid was a fair fixing agent and better than stronger solutions.

The fact that for so many of these substances the weaker solutions were better fixing agents than stronger ones, leads to the belief that combinations of solutions much weaker than those commonly employed should be developed. They may be more satisfactory as fixing agents as well as being much more economical.

Formalin is not a satisfactory fixing agent for protozoa when nuclear details are of importance. Five per cent was not strong enough to coagulate fecal smears so that they adhered to the coverglasses. Stronger

solutions produced much distortion. Formalin does, however, definitely increase the reactivity of the peripheral layer of the nucleus of *Endolimax* for the Feulgen reaction as do also to a less extent, hydrochloric and nitric acids.

Approximately one hundred new combinations of mercuric chloride, alcohol, acetic acid, picric acid, chromic acid, with other substances added have been tried out as fixing agents, and while many of them have proven to be excellent fixing agents for protozoa, none of them appeared to be superior to Schaudinn's fluid, or its dilutions.

In the discussions which follow, the acetic acid used was glacial acetic. Hydrochloric, nitric, and sulfuric acids were Merck's C.P. diluted to the strength indicated. Unless otherwise stated, the stain referred to was Heidenhain's iron alum hematoxylin.

THE MORPHOLOGY OF CERTAIN SPECIES IN RELATION TO TECHNIQUE

Trichomonas muris

Reference has already been made to the fact that *T. muris* usually does not show a parabasal body when stained with Heidenhain's after fixation with Schaudinn's fluid. (In a few cases, however, I have seen the parabasal body of *T. augusta* when preparations were made in the same way.) After fixation with Flemming's fluid, or chrom-acetic, Heidenhain's may stain the parabasal. Cutler (1919) reported similar results with *Ditrichomonas termitis*. In 1926 Grassé discussed the literature up to that time and the results of studies by himself and his associates and believed that acetic acid is especially destructive to the parabasal body. Kirby (1931) reviewed the previous literature and showed that the parabasal body of trichomonad flagellates is stainable with Delafield's after fixation with Schaudinn's fluid, although not stainable with Heidenhain's. The difference in staining reaction of *T. muris* as between Delafield's and Heidenhain's stains after fixation with Schaudinn's fluid is even more striking than the statements reviewed above indicate. After staining with Heidenhain's, one sees that the blepharoplast, costa, chromatic margin of the undulating membrane, nuclear chromatin and various zones of cytoplasmic granules all stain intensely (Fig. 1). When stained with Delafield's, however, the costa, chromatic margin of the undulating membrane, and the parabasal body all stain deeply while the cytoplasmic granules are not stained at all (Fig. 2). After fixation in Schaudinn's without acetic, the parabasal did not stain nearly so well with Delafield's. When the same material was fixed with one per cent acetic alone and stained with Heidenhain's, only the endosome and the costa stained black; with Delafield's, the costa and peripheral layer of the nucleus stained very well but the parabasal was very poorly stained. When this material was fixed in 20 per cent acetic acid alone and stained with Heidenhain's, noth-

ing stained intensely and there was a wide vacant streak near the dorsal profile (Fig. 3). When Delafield's was applied to such a smear, the parabasal stained more intensely than any other structure (Fig. 4). It is therefore obvious that acetic acid does not destroy the parabasal body, at least in the strength of 20 per cent used alone as a fixing agent. When this same *T. muris* material was fixed in 2 per cent chromic acid containing 5 per cent of acetic acid and stained with Heidenhain's, the endosome of the nucleus and the costa were the only structures that stained intensely (Fig. 5), and the same was true for the slide stained with Delafield's. After Zenker's fluid as a fixative, Delafield's did not stain the parabasal body but did stain the central region of the axostyle, which does not ordinarily take a stain. It seems obvious, therefore, that the apparent morphology of *T. muris* varies widely depending upon the techniques to which it has been subjected.

Giardia lamblia

Although I have conducted a good many experiments with other kinds of trichomonad flagellates, limitations of time and space will not permit of their being discussed in this place. I should, however, like to refer to a few experiments with *Giardia lamblia*. Cysts of this species seem to be singularly uninfluenced by various fixing agents as judged by subsequent staining with iron hematoxylin. After most of the fixing solutions and their modifications that I have tried, there was little variation in the usual picture of a deep stain for the endosomes of the nucleus and for the various parts of the fibrillar apparatus. There were, however, differences in the amount of shrinkage inside the cyst membrane. For example, Schaudinn's without acetic gave rise to more intracystic shrinkage than when 2 to 15 per cent of acetic acid was added. In one batch of material fixation in chrom-acetic with a little copper oxide added produced practically no shrinkage of the protoplasm within the cyst membrane, in contrast to the usual fixing agents, but nothing in the cell stained with Heidenhain's stain. In another series fixation was made with Schaudinn's plus 5 per cent of acetic acid and with 5 and 25 per cent of acetic acid alone. For each of these the staining reaction with iron hematoxylin was practically the same, in the manner illustrated in Fig. 9. With the 25 per cent acetic fixation, the peripheral layer of the nucleus stained in some cysts. For each fixative, a smear was also stained in phosphotungstic acid hematoxylin. With this stain the peripheral layer against the nuclear membrane stained very well after Schaudinn's plus 5 per cent acetic, while the endosomes did not (Fig. 10). After the 25 per cent acetic acid fixation, however, the endosomes stained as well as the peripheral layer.

As reported by Lucas (1930) and Levitanskaia (1938), the endosomes of cyst nuclei nearly always give a strong positive reaction to the

Feulgen technique. They also stain strongly with hemalum, which, however, does not stain the fibrillar structures well.

Reference was made above to the differences in stainability with Heidenhain's on the same slide, when, of necessity, all phases of technique are the same. Such differences are illustrated in Figs. 6, 7, 8, and 9, each drawn from the same slide from regions destained about the same amount. In many cases, therefore, the variations for the same technique are greater than for different techniques. It is, therefore, necessary to exercise great caution in interpreting the results of different techniques.

Iodamoeba bütschlii

Since the genera of endozoic amoebae, and species also within the genus *Entamoeba*, are differentiated to a large extent by the structure of the nuclei, the effects of different fixing agents and stains on the appearance of nuclei has been given primary consideration in these experiments and receives attention in the following pages and the accompanying illustrations.

In a previous paper (1937b) attention was called to the fact that higher percentages (10 to 20 per cent) of acetic acid added to Schaudinn's fluid tended to reduce the stainability of the endosome in the cysts of *Iodamoeba bütschlii*. This tendency is subject to a considerable amount of variation. Fig. 11, for example, shows a cyst fixed in Schaudinn's plus 10 per cent acetic and stained with iron hematoxylin. The endosome does not look as solid as it ordinarily does if only 2 or 5 per cent acetic has been added. In many cysts on this same slide the endosomes were unstained. However, in one case no essential difference was noted in smears fixed in Schaudinn's fluid to which 5, 10, and 20 per cent of acetic acid had been added, while in another case all the endosomes were unstained when 20 per cent of acetic had been added to Schaudinn's. Schaudinn's plus 5 per cent acetic reduced to one-half and to one-quarter strength produced about the same result as the full strength solution.

When 1 per cent HCl, 5 per cent HNO₃, 1 per cent urea and 5 per cent urea were each added to Schaudinn's fluid and tested separately, they all gave somewhat similar results so far as nuclear staining was concerned, the endosomes staining intensely black with Heidenhain's. There were differences in the effects on the cytoplasm.

When saturated mercuric chloride solution was used alone, cytoplasmic fixation was fair but the nucleus tended to stain a homogeneous black. About the same results were obtained with 75 and 50 per cent of a saturated solution. With 25 per cent and 10 per cent of saturation, however, cytoplasmic fixation and nuclear differentiation were much better. When hemalum was used as the stain after 50 per cent of saturated mercuric chloride, the lightly stained endosome often contained a small deeply

stained granule (Fig. 12). Such a granule was noted in the paper on *Iodamoeba* already published, again after hemalum staining, but after Schaudinn's plus 10 and 20 per cent of acetic acid as the fixative. It is possible that this granule represents a "centriole" or division center.

Alcohol of a strength of 50 per cent produced less distortion and shrinkage of the cytoplasm of cysts and better differentiation of nuclei than 70 and 95 per cent. With hemalum the nuclear staining was about the same for all three strengths. When different strengths of acetic acid were used alone as fixing agents, they produced a good deal of cytoplasmic distortion, the amount increasing with increasing strengths. After 2 and 5 per cent the nuclei stained very well with black endosomes but after 10 per cent the staining ability of the endosomes decreased (Fig. 13), and after 20, 25, and 50 per cent acetic the nuclei remained unstained.

Saturated and 50 per cent of saturated picric acid fixed the cytoplasm very well, but the nuclei tended to overstain.

Bouin's fluid and several modifications of it were satisfactory fixation agents for trophic stages but produced much distortion of the cytoplasm and variability of staining in the nuclei of cysts. Alcoholic Bouin's was equally unsatisfactory for cysts and even produced cytoplasmic extrusions or "buds." Picromercuric, on the other hand, proved to be a very satisfactory fixing agent for *Iodamoeba* cysts.

Endolimax nana

Endolimax nana, as reported by Stabler (1932a), usually shows a loss of stainability of the endosome and an enhanced stainability of the peripheral layer of the nucleus with Heidenhain's staining after Schaudinn's with 15 to 20 per cent of acetic acid added. Figs. 14 and 15 show the trophic stage and cyst as they commonly appear after fixation with Schaudinn's plus 5 per cent of acetic acid added. Figs. 16 and 17 show the corresponding stages from the same fecal sample fixed with Schaudinn's plus 20 per cent acetic. The pronounced loss of stainability of the endosome is the typical condition. However, this reaction is subject to considerable variation. Figs. 18, 19, and 20 are all from a slide fixed in Schaudinn's with no acetic acid added. The great majority of the amoebae showed stained endosomes but some showed the endosomes unstained. Figs. 21, 22, and 23 are all from a slide from the same material fixed in Schaudinn's plus 20 per cent acetic. On this slide the majority showed stainless endosomes (Figs. 22, 23) but in some the endosomes stained deeply (Fig. 21). Addition of only 2 per cent of acetic acid has proved better than higher concentrations in some cases but not so good in some others. Schaudinn's plus 20 per cent acetic sometimes produces "buds" on cysts of *Endolimax*. Dilution of Schaudinn's did not produce any differences in stainability of the trophic stages.

Figs. 26, 27, and 28 are, respectively, from slides fixed in full strength, 50 per cent, and 25 per cent of the same. There were no noticeable differences in the results from the three strengths of this fixative.

When mercuric chloride was used alone in a saturated solution and in 75, 50, and 25 per cent of saturation, the weaker solutions proved to be better fixing agents than the stronger ones.

Acetic acid alone tends to restrict the stainability of the nuclei in both the trophic and cyst stages when stained in Heidenhain's. Even one per cent may have some effect in this direction and 20 per cent is followed by loss of stainability. Combined with other substances, acetic acid may have other effects. Figs. 29 to 38 all from the same source material, illustrate some results obtained by using mercuric chloride and alcohol with and without acetic acid, upon the stainability of the nuclei of trophic stages. After saturated mercuric (Fig. 29), the nuclei stain fairly well but not as darkly as when 5 per cent of acetic acid is added (Fig. 30). In this case, acetic added to the sublimate intensifies the stainability of the endosome. When 50 per cent of saturated mercuric chloride is used (Fig. 31), the endosome stains more intensely than when 5 per cent of acetic acid is added to it (Fig. 32). In the latter case, the tendency for the acetic to cause lighter staining of the endosome is effective against the weaker sublimate solution. When 50 per cent alcohol is used alone (Fig. 33), the endosome stains very black, but when 5 per cent acetic is added to it some of the stainability is lost (Fig. 34). Similar effects are seen for 70 per cent alcohol (Fig. 35) and the same with 5 per cent acetic added (Fig. 36). But when 95 per cent alcohol is used (Fig. 37), the endosome is apparently prevented from showing any loss upon the addition of acetic (Fig. 38). The effect of 5 per cent acetic by itself is shown in Figs. 39 and 40, where a somewhat lighter stain is obtained by differentiating with iron alum (Fig. 39) than with picric acid (Fig. 40).

Other acids, hydrochloric, nitric, and sulfuric in concentrations of 5 and 10 per cent of the C.P. solutions produced much distortion of protoplasm and variability in the staining capacity of the nuclei. Nitric acid was followed by better nuclear differentiation than either of the others.

Bouin's fluid sometimes produces shrinkage areas between the nuclei and surrounding cytoplasm in trophic stages (Fig. 25). It usually produces considerable shrinkage and distortion within cysts and irregularity of stainability of the nuclei. Picric acid in the one case when it was tried produced a fair result of both fixation and staining of trophic stages.

Segal (1932) has called attention to the fact that "buds" were produced on the cysts of *E. nana* more frequently when they were fixed in picromercuric than when fixed in Schaudinn's fluid and several other fixing agents. She also noted that different strains of *E. nana* had dif-

ferent tendencies in regard to the formation of "buds." The present study has confirmed the work of Segal. "Buds" were also produced to some extent by 25 and 50 per cent acetic acid.

Both hemalum and phosphotungstic acid hematoxylin, after most of the fixatives used, had a greater affinity for the peripheral layer of the nucleus than Heidenhain's hematoxylin.

Dientamoeba fragilis

Both Johnson (1935) and I (Wenrich, 1937a) have called attention to the fact that the nuclei of *Dientamoeba* do not usually stain well with Heidenhain's when fixed with Schaudinn's fluid unless the percentage of acetic added is as high as 15 or 20. This result has been confirmed many times but it has exceptions. For example, in some cases, when nuclei failed to stain after Schaudinn's plus 5 per cent acetic (Fig. 53), they stained very well when the same material was fixed in the same fixing solution reduced to one-half strength (Fig. 54); and in some other cases Schaudinn's plus 2 per cent acetic gave better staining than Schaudinn's plus 5 per cent, as did also this solution with 20 per cent acetic added. In still other cases the nuclei stain very well when the feces are fixed in Schaudinn's without acetic (Fig. 55). The feces represented by Fig. 55 was strongly acid to litmus paper at the time that the fixation was made. It is therefore suspected that various organic acids may enhance the stainability of the nucleus to Heidenhain's when sublimate-alcohol is the basic fixing substance.

The effect of acetic acid in combination with sublimate and alcohol is illustrated by Figs. 41–50; these figures are from the same slides that are represented in Figs. 29–38. Figs. 29 and 41 are from the same slide, as are Figs. 30 and 42, and so on through the series. Fig. 41 shows the nuclei barely discernible after fixation with saturated sublimate alone. When 5 per cent of acetic acid is added to this the nuclei stain very well (Fig. 42). With 50 per cent of saturated sublimate the nuclei are undifferentiated (Fig. 43), but with the addition of 5 per cent acetic they are well stained (Fig. 44). With 50 per cent alcohol the nuclei differentiate poorly (Fig. 45); but with the addition of 5 per cent acetic the nuclei stain distinctly. With 70 per cent alcohol (Fig. 47) the nuclei stain but the intranuclear granules are less distinct than when 5 per cent acetic is added (Fig. 48). The story is again similar for 95 per cent alcohol (Fig. 49) and the same strength with 5 per cent acetic added (Fig. 50). The effect of 5 per cent acetic alone is illustrated by Figs. 51 and 52. These are from the same slides as Figs. 39 and 40 and show no significant difference as between alum differentiation (Fig. 51) and picric acid destaining (Fig. 52).

This group of figures (Figs. 39 and 40; 51 and 52) shows the con-

trast between *Endolimax* and *Dientamoeba* in relation to acetic acid; this acid reduces the stainability of the central area of *E. nana*, but increases the stainability of the central area of *Dientamoeba*. Acetic acid of any strength appears to fix the nuclei of *Dientamoeba* so that they will stain more readily. Fig. 56 represents an individual from a slide fixed in full strength glacial acetic acid. The nuclei are fairly well stained but the granules of the central area are not too well differentiated.

It has been mentioned that Bouin's fluid, especially the B3 modification, is usually followed by excellent staining of the nuclei of *Dientamoeba*. Other fixing agents which give fairly satisfactory results are Hollande's, Kleinenberg's, Carnoy No. 2, Zenker's, and weak Flemming's. Hemalum is an excellent stain for *Dientamoeba*.

Entamoeba histolytica

As previously reported (1938), Schaudinn's fluid plus 5 per cent of acetic acid, or the same solution diluted with an equal quantity of distilled water, is about the most satisfactory fixing agent for *E. histolytica* that I have found thus far. Even one-fourth strength of this common fixative resulted in satisfactory fixation.

When different strengths of sublimate were used alone, the weaker solutions of 10, 25, and 50 per cent of a saturated solution gave better fixation than 75 or 100 per cent of saturated solution. Alcohol at 50 per cent gave better fixation and less distortion of the cytoplasm than 70 or 95 per cent, although nuclear staining was similar in all. In one case the use of 50 per cent resulted in the formation of a number of "buds" on cysts while none occurred when 95 per cent was used.

The stainability of the nuclei in Heidenhain's is much affected by acetic acid. If 20 per cent or more is added to Schaudinn's fluid or to other sublimate alcohol combinations, the nuclei tend to lose stainability, but the results vary with different cases. In one case where there were cysts of the small race of *E. histolytica*, the nuclei failed to stain when the smears were fixed in Schaudinn's without acetic. Nuclear stainability gradually increased with addition of 2, 5, and 10 per cent of acetic acid but diminished with the addition of 20 per cent.

When acetic acid was used alone even in strengths as low as 1 or 2 per cent, the stainability of the nuclei in Heidenhain's was diminished. One series of slides from the same material and stained with Heidenhain's is illustrated by Figs. 57 to 67. Figs. 57 and 58 show trophic and four-nucleated cysts from the same smear fixed in Schaudinn's fluid plus 5 per cent of acetic. The usual staining picture is revealed. When 5 per cent acetic alone was used as the fixative, the endosomes failed to stain while the peripheral layer of the nuclei retained its stainability (Figs. 59, 60). When 10 per cent acetic was used, the stainability was still more reduced

although retained better in the cysts than in the trophic stages (Figs. 61, 62). With 20 per cent acetic practically all stainability was removed from the nuclei (Figs. 63, 64). With 50 per cent acetic as the fixing agent, some stainability returned to the peripheral layer of the nuclei (Figs. 65, 66), but especially in the trophic stage (Fig. 65). Most of the cysts showed a relatively large shrinkage space between the cytoplasm and the cyst wall with this last fixative, and some of them showed relatively extensive "buds" protruding through breaks in the cyst membrane. These protoplasmic extrusions gave the appearance of having been produced explosively and the outer boundary was indefinite (Fig. 67). This type of "bud" is unlike those produced by other agents, such as Schaudinn's plus 5 per cent acetic (Fig. 68).

Bouin's fluid usually results in much intracystic distortion and irregularity of stain in the nuclei and in shrinkage spaces inside or outside the nuclei of trophic stages (Fig. 70). In many cases the endosomes fail to stain in the trophic stages after the use of B3 (Fig. 72). In this series of slides a very small endosome, perhaps the centriole, was usually visible when one per cent of urea was added to B3. Alcoholic Bouin's gives much the same results except that "buds" are more likely to be produced on the cysts. In one case 25 to 30 per cent of the cysts showed "buds" after the use of alcoholic Bouin when not more than 2 per cent were seen in the same material fixed with B3 and one-half strength B3. In this same material picromercuric produced between 45 and 50 per cent of "buds."

Entamoeba coli

Anyone who has examined any considerable number of series of slides showing *E. coli* cysts from different hosts is well aware of the variability seen when fixation is in Schaudinn's fluid and the stain is iron hematoxylin. In some cases there will be a high percentage of cysts that will be unstained; in others there may be a high percentage of cysts that are over-stained; in still others there may be high percentages of both unstained and overstained cysts. In some cases there may be "buds" on some of the cysts and in other cases there will be none. Because of the variability of the organism itself, it is difficult to obtain consistent differences with different techniques.

Higher percentages of acetic acid added to Schaudinn's fluid have less tendency to diminish the stainability of the nuclei than with *E. histolytica*. Such loss of stainability that may occur affects the endosome more readily than the peripheral layer.

In one race which showed about 30 per cent "buds" on the cysts after fixation with Schaudinn's plus 5 per cent acetic, there were no "buds" when sublimate alone was used either in saturated or 50 or 25 per cent

of saturation. When the same material was fixed in 5, 10, 25, and 50 per cent of acetic acid, nuclear staining diminished from the 10 up to the 50 per cent acetic. No "buds" were seen after 5 and 10 per cent, but about 20 per cent of cysts showed "buds" after 25 per cent acetic and 90 per cent showed "buds" after the 50 per cent. In another case where 2 and 100 per cent glacial acetic were used as fixatives, there was scarcely any distortion after the 2 per cent and the nuclei stained very well, except that in the trophic stage the endosomes failed to stain in most of the nuclei. After the 100 per cent acetic there was usually a very pronounced shrinkage space between the cyst wall and the protoplasm on the cysts and in some the protoplasm was unstained and in others it was homogeneous black. In some cysts there were the same type of "buds" as seen in the other case cited above. In the trophic stages there was some stainable material in the periphery of the nuclei but none in the central area. The extrusions or "buds" produced by 50 and 100 per cent acetic were relatively enormous and gave the appearance of having been produced explosively. They usually failed to show any definite boundary layer making it difficult to recognize the contours. In this respect they were like the similar extrusions produced by 50 per cent acetic in *E. histolytica* (Fig. 57).

There seems to be fairly general agreement at the present time that Kofoid and Swezy's (1921) "*Councilmania lafleuri*" is only a variation of *E. coli* and that the "buds" and chromophile ridges described by these authors and considered by them to be diagnostic for "*C. lafleuri*" are produced by the agents commonly used in fixation. Freeman (1929) working in my laboratory, found that Yocum's picromercuric was highly effective in producing "ridges" and "buds." Stabler (1932b) reported that 95 per cent alcohol at 55 degrees C and picromercuric at 28 degrees were more effective in producing "buds" than the other agents tried. We can now add strong acetic acid to the list of effective "bud"-producing agents. The production of "buds" is a highly variable phenomenon, however, and these variations indicate either, (1) that certain races produce "buds" more readily than others or, (2) that "budding" occurs more readily in the same race under certain conditions presented by the surrounding feces than in other conditions. Probably a combination of these two possibilities is nearer the truth.

DIFFERENT SPECIFIC REACTIONS TO THE SAME TECHNIQUES

As previously pointed out the genera of endozoic amoebae have been differentiated from each other primarily on the basis of nuclear structure. A study of their reactions to varying techniques shows that these nuclei are not only different in organization but different in their chemical properties as reflected in different reactions to the same technique. An

attempt has here been made to demonstrate such differences by illustrating the results when the different species have been fixed the same way (in this case in Schaudinn's plus 5 per cent of acetic acid) but subjected to three different staining techniques.

On Plate IV Figs. 73–81 are from slides stained with Heidenhain's; Figs. 82–90 with hemalum and Figs. 91–99 with the Feulgen technique. All the figures of *E. coli*, *E. histolytica* and *Endolimax nana* are from the same source. Figs. 73–76, 79, and 80 are from the same slide; Figs. 82–85, 88 and 89 are from another slide from the same source; and Figs. 91–94, 97 and 98 are from still another slide of the same series. Figs. 77, 78, 86, and 87 are from one source, and Figs. 81 and 90 are from this same source. Only the material giving a deep staining reaction has been drawn in detail; this includes the chromatoids of *E. coli* and *E. histolytica*. In the drawings from the Feulgen slides the nuclear boundaries and other non-staining nuclear areas have been outlined only.

With iron hematoxylin the endosomes and the peripheral layer of granules stain deeply in *E. coli* and *E. histolytica*. In *Endolimax nana* the peripheral layer may or may not be readily discernible after this stain. With *Iodamoeba* the endosomes stain intensely as do also the periendosomal granules. In *Dientamoeba* the central nuclear area usually contains four or more deeply stained granules imbedded in a less deeply stained matrix.

With hemalum, the peripheral layer usually stains better than the endosomes in the species of *Entamoeba*. In *Iodamoeba* the periendosomal granules stain better than the endosome. In *Endolimax* the endosome normally stains well as does also the peripheral layer. In *Dientamoeba* the staining reaction is comparable to that with Heidenhain's. In *E. histolytica* the failure of the endosome to stain with hemalum is more often noted than with *E. coli*, but is not always observed in either species. In both species it will be noted that there is a certain amount of deeply stained material in the immediate vicinity of the endosome. This material is more voluminous in *E. coli*, making it more difficult to tell whether the endosome proper has been stained (Figs. 82, 83).

The contrast between the nuclear picture after the Feulgen technique and the other stains is very striking. In *E. coli* and *E. histolytica* only the material in the vicinity of the endosome shows the reaction (Figs. 91–94). In some cases where Fast Green was used as the counter stain, a small green granule could be seen amongst the Feulgen reacting granules, in the central nuclear area. In the cysts of *E. coli* the reacting material is often scattered (Fig. 92) as it often is also in the trophic stage (Fig. 91). In *Iodamoeba* the endosome shows no reaction but the zone of periendosomal granules reacts strongly. In *Endolimax* the endosome of the trophic stage shows no reaction but the peripheral layer gives a

weak reaction. This reaction may be intensified by the use of formalin as a fixing fluid and it is also somewhat intensified in the cysts, at least, by hydrochloric and nitric acids. In *Dientamoeba* the appearance of the nuclei is not essentially different from what it is with the other two stains. In this respect this species is distinctly different from all the others.

In some respects the Feulgen reactions recorded here are different from those recorded by certain other observers. For example, Levitanskaia (1938) reports that *E. coli* has a large reacting karyosome (endosome) in the trophic stage. In the cysts he reports scattered granules as I have found. Both Reichenow (1928) and Levitanskaia state that there is a faint reaction in the peripheral layer of *E. histolytica* but I have not been able to confirm this. Both these authors reported the same results with *Iodamoeba* that I have obtained. Levitanskaia noted the faint reaction of the peripheral layer in the nucleus of *Endolimax* trophic stages but stated that in the cysts only the endosome reacts. I have found the cyst reaction to be variable; some show a reaction in the peripheral area as shown in Fig. 98 while others show a reaction in the central area. It seems probable that when the reacting material appears in the central area, some phase of nuclear division, either prophase or telophase, is involved. My results with *Dientamoeba* agree with those of Levitanskaia.

On the whole, these evidences of chemical differences, especially as between and among the different genera offer important confirmation to the validity of the taxonomic groupings which have been established.

SUMMARY

1. The importance of properly prepared slides as an aid to the diagnosis of intestinal protozoa is emphasized.
2. Experiments show that fixation for one minute gives results as good as or better than longer times for the fixing agents commonly employed for intestinal protozoa.
3. Schaudinn's fluid and other fixing solutions can be greatly diluted without destroying their effectiveness as fixing agents. Mercuric chloride, acetic acid, alcohol, etc., in weaker solutions, are better fixing agents than stronger solutions. It is suggested that new fixing agents, much weaker than those commonly employed, may well be devised.
4. Acetic acid does not destroy the stainability of the parabasal body of *Trichomonas muris* for hemalum even when used alone at 20 per cent. Strong concentrations do reduce the stainability of the nuclei of the common intestinal amoebae except *Dientamoeba*. It tends to increase the stainability of the nuclei of that species. Strong concentrations (50 per cent or more) often produce relatively large "buds" on the cysts of *E.*

histolytica and *E. coli*. These "buds" seem to lack definite boundaries at their outer margins.

5. As with other organisms, intestinal protozoa sometimes show a surprising amount of variation. Different races of the same species may have different appearances with the same technique. The same race may give different results with the same technique, if the environments are different. Different individuals on the same slide may show different reactions to the same technique.

6. On the other hand, as illustrated by *Trichomonas muris*, different fixing solutions and different stains may produce decidedly different appearances for the same species.

7. Different species show chemical as well as morphological differences in the composition of their nuclei. This is particularly well shown when the Feulgen reaction is applied to the intestinal amoebae of man and the results compared with those obtained with the more common stains.

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EXPLANATION OF PLATES

All figures have been drawn from fixed and stained slides. Unless otherwise stated, the stain has been Heidenhain's iron alum hematoxylin. Fixing agents are mentioned in the detailed explanations. Figs. 1 to 10 were drawn originally at $\times 4000$. All others at $\times 3000$. All have been reduced about one-third in reproduction.

PLATE I

FIGS. 1 to 5. *Trichomonas muris*, all from the same rat.

FIG. 1. Schaudinn's plus 5% acetic (Hendemann). Note deeply stained floccular apparatus and cytoplasmic granules.

FIG. 2. Schaudinn's plus 5% acetic; Delafield's. Parahyal stained granules unstained.

FIG. 3. 20% acetic (Hendemann). Floccular apparatus smaller but unstained.

FIG. 4. 20% acetic; Delafield's. Parahyal stained.

FIG. 5. 2% chromic acid plus 5% acetic (Hendemann). Endosome and cysts are only structures stained.

FIGS. 6 to 10. Cysts of *Giardia lamblia*.

FIGS. 6 to 9. Schaudinn's plus 5% acetic. All from one slide. Note variations in staining.

FIG. 10. Schaudinn's plus 5% acetic; phosphotungstic acid hematoxylin. Peripheral layer of nucleus stains deeply, endosomes do not.

FIGS. 11 to 13. Cysts of *Iodamoeba bütschlii*.

FIG. 11. Schaudinn's plus 10% acetic. Endosome stained. Microsome areas, endosomes are usually unstained.

FIG. 12. 50% of sat. H_2CO_3 treatment. Endosome faint except for small granule (=centriole?).

FIG. 13. 10% acetic. Very little stain, endosome not clearly differentiated.

FIGS. 14 to 23. *Endolimax nana*.

FIGS. 14 to 17 are from same case.

FIGS. 14 and 15. Schaudinn's plus 5% acetic. Endosomes deeply stained.

FIGS. 16 and 17. Schaudinn's plus 20% acetic. Endosomes faint.

FIGS. 18 to 23 are from another case.

FIGS. 18 to 20. Schaudinn's without acetic. Variable stainability of endosome on same slide.

FIGS. 21 to 23. Schaudinn's plus 20% acetic. Another slide, variable stainability of endosome.

FIGS. 24 and 25. Another case.

FIG. 24. Schaudinn's plus 5% acetic. Appearance typical.

FIG. 25. Eosin. Surrounding space around nucleus.

FIGS. 26 to 28. Another case. Fixations are, respectively, Schaudinn's plus 5% acetic, one-half the same, one-fourth the same.

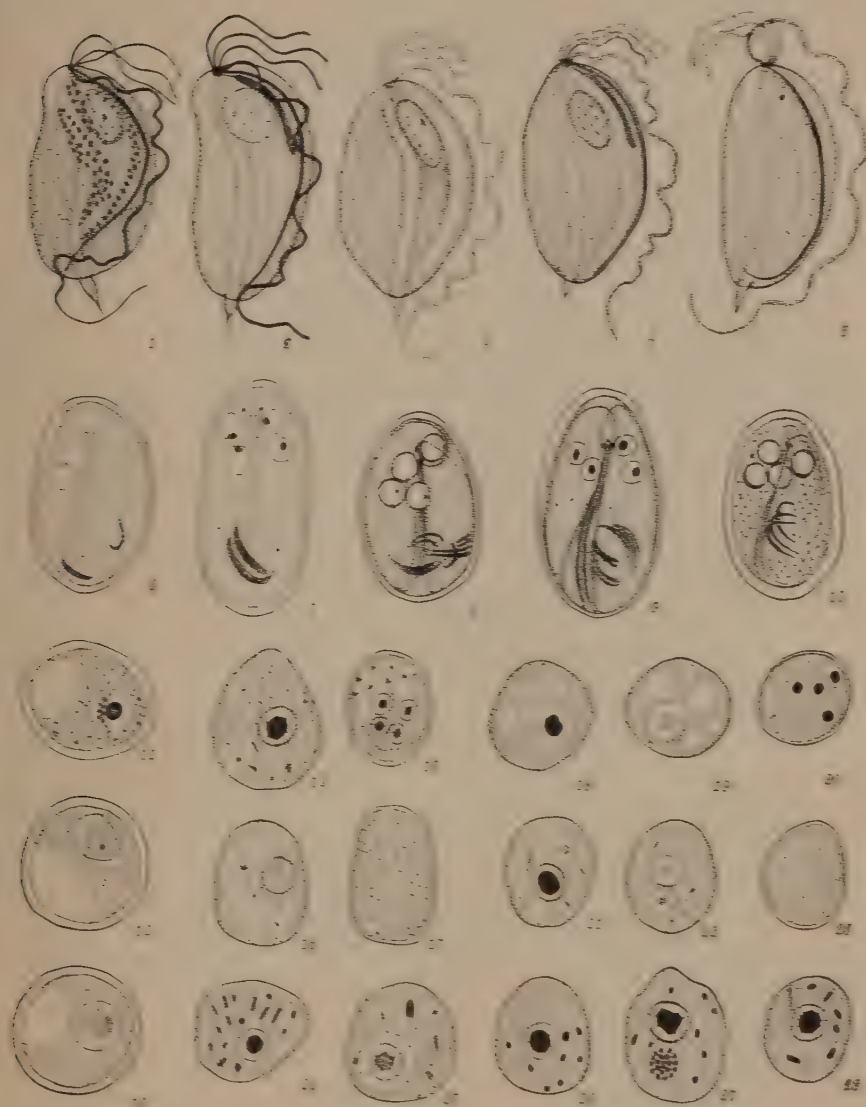


PLATE I

PLATE II

FIGS. 29 to 40. *Endolimax nana* trophic stages, all from same case; all differentiated with picric except Fig. 39.

FIG. 29. Sat. HgCl_2 . Endosome not deeply stained.

FIG. 30. Same plus 5% acetic. Endosome deeply stained.

FIG. 31. 50% of sat. HgCl_2 . Endosome deeply stained.

FIG. 32. Same plus 5% acetic. Endosome lightly stained.

FIG. 33. 50% ethyl alcohol. Endosome darkly stained.

FIG. 34. Same plus 5% acetic. Endosome more lightly stained.

FIG. 35. 70% ethyl alcohol. Endosome darkly stained.

FIG. 36. Same plus 5% acetic. Endosome more lightly stained.

FIG. 37. 95% ethyl alcohol. Endosome darkly stained.

FIG. 38. Same plus 5% acetic. Endosome darkly stained.

FIG. 39. 5% acetic acid. Alum differentiation. Endosome lightly stained.

FIG. 40. Same, picric differentiation. Endosome darker.

FIGS. 41 to 56. *Dientamoeba fragilis*. All picric differentiation except Figs. 51, 53, 54, 55.

FIGS. 41 to 52. Same slides as Figs. 29 to 40. Figs. 29 and 41 from same slide; Figs. 30 and 42 from same slide, etc.

FIG. 41. Sat. HgCl_2 . Nuclei indistinct.

FIG. 42. Same plus 5% acetic. Nuclear granules stained.

FIG. 43. 50% sat. HgCl_2 . Nuclei and nuclear granules indistinct.

FIG. 44. Same plus 5% acetic. Nuclear granules stained.

FIG. 45. 50% ethyl alcohol. Nuclear granules indistinct.

FIG. 46. Same plus 5% acetic. Nuclear granules distinct.

FIG. 47. 70% ethyl alcohol. Nuclei fairly well differentiated.

FIG. 48. Same plus 5% acetic. Nuclear granules more distinct.

FIG. 49. 95% ethyl alcohol. Central nuclear area stained but granules indistinct.

FIG. 50. Same plus 5% acetic. Nuclear granules more distinct.

FIGS. 51 and 52. 5% acetic; alum and picric differentiation, respectively; no significant difference.

FIGS. 53 and 54. Another case.

FIG. 53. Schaudinn's plus 5% acetic. Nuclei poorly differentiated.

FIG. 54. Same, one-half strength. Nuclei more clearly differentiated.

FIGS. 55 and 56. Another case; feces acid.

FIG. 55. Schaudinn's with no acetic. Nuclei fairly well stained.

FIG. 56. 100% acetic. Central nuclear area stained, granules indistinct.

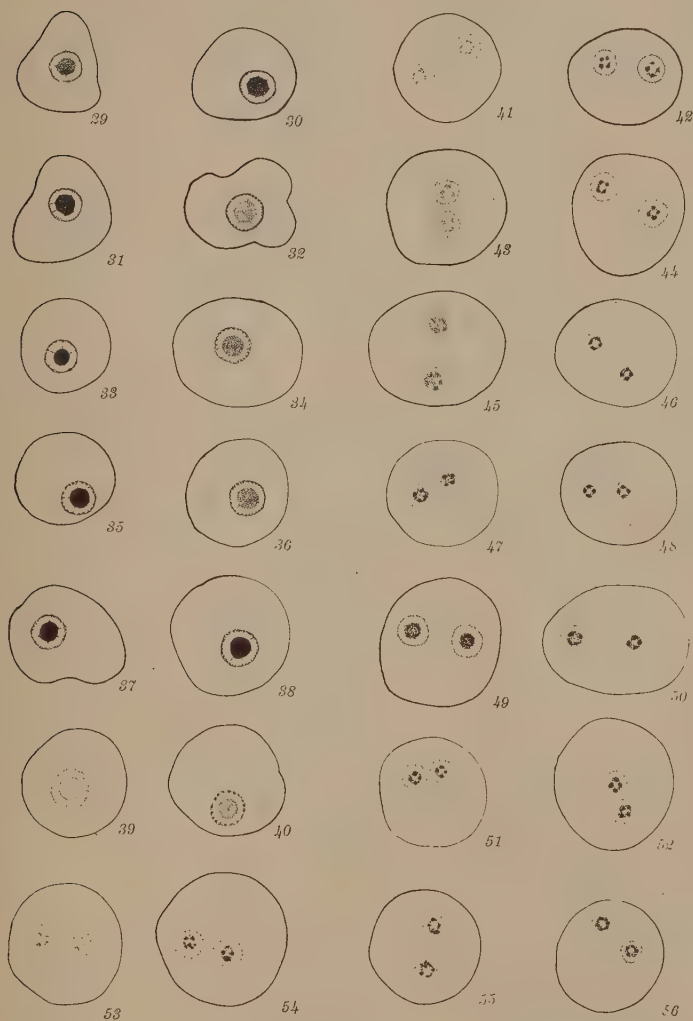


PLATE II

PLATE III

FIGS. 57 to 72. *Entamoeba histolytica*.

FIGS. 57 to 67. From same case; picric differentiation.

FIGS. 57 and 58. Schaudinn's plus 5% acetic. Trophic stage and mature cyst, typical staining.

FIGS. 59 and 60. 5% acetic. Trophic stage and cyst. Endosome unstained.

FIGS. 61 and 62. 10% acetic. Trophic stage and cyst. A little stain remains in peripheral layer of nucleus.

FIGS. 63 and 64. 20% acetic. Trophic stage and cyst. (Note parasitic *Sphaerita* in cyst.) No stain in nuclei.

FIGS. 65 and 66. 50% acetic. Trophic stage and cyst. A little stain in peripheral layer of trophic stage; broad shrinkage space in cyst.

FIG. 67. 50% acetic. Cyst. Broad extrusion ("bud"), with no definite boundary on outer surface.

FIG. 68. Schaudinn's plus 5% acetic. Another case. A typical "bud" with definite boundary.

FIGS. 69 and 70. Another case, trophic stages.

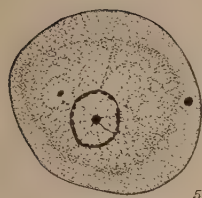
FIG. 69. Schaudinn's plus 5% acetic. Typical condition of nucleus.

FIG. 70. Allen's B3. Shrinkage area inside nucleus.

FIGS. 71 and 72. Another case, trophic stages (drawings by Mr. R. L. Brown).

FIG. 71. Schaudinn's plus 5% acetic. About usual condition.

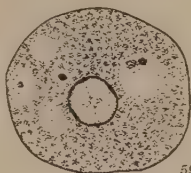
FIG. 72. Allen's B3. Endosome unstained. Central nuclear area shows contraction.



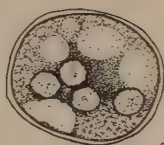
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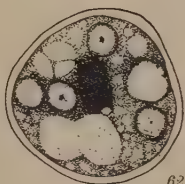
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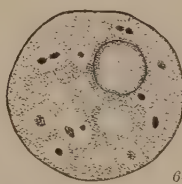
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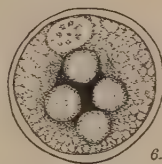
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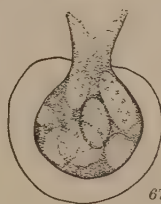
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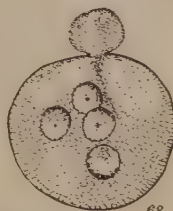
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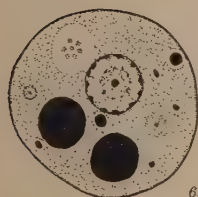
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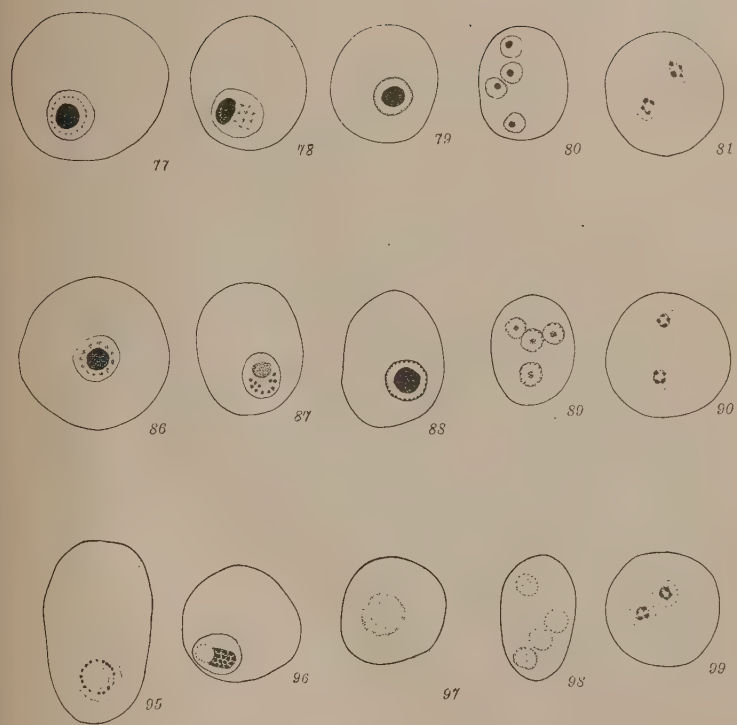
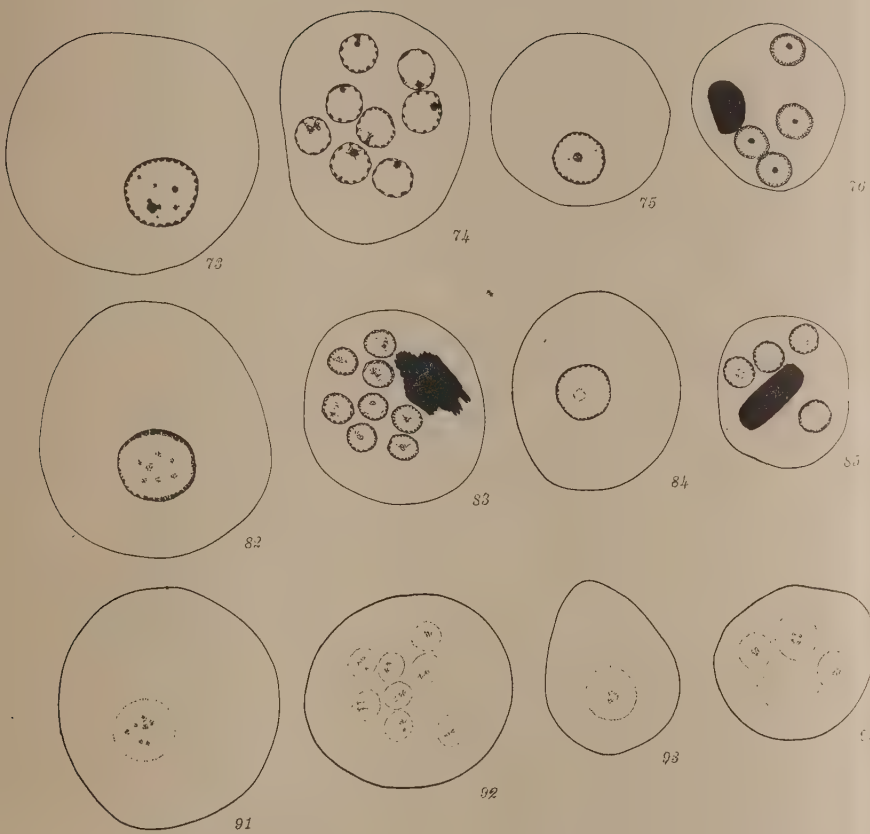


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PLATE IV

The intestinal amoebae of man. Fixed in Schaudinn's plus 5% acetic. Aside from nuclear and endosomal profiles only deeply stained nuclear materials and chromatoids were drawn. For Figs. 73 to 81, stain is Heidenhain's; Figs. 82 to 90, stain is hemalum; Figs. 91 to 99, stain is Feulgen. Figs. 73, 74, 75, 76, 79, 80 are from one slide; Figs. 82, 83, 84, 85, 88, 89 are from another slide from same case; Figs. 91, 92, 93, 94, 97, 98 are from a third slide from this case. Figs. 77 and 78 are from one slide from another case. Figs. 86 and 87 are from another slide from this case. Figs. 95 and 96 are from different cases. Figs. 81 and 90 are from one case; Fig. 99 from another case.

- Figs. 73, 82 and 91. Trophic stages of *E. coli*.
- Figs. 74, 83 and 92. Cysts of *E. coli*.
- Figs. 75, 84 and 93. Trophic stages of *E. histolytica*.
- Figs. 76, 85 and 94. Cysts of *E. histolytica*.
- Figs. 77, 86 and 95. Trophic stages of *Iodamoeba bütschlii*.
- Figs. 78, 87 and 96. Cysts of *I. bütschlii*.
- Figs. 79, 88 and 97. Trophic stages of *Endolimax nana*.
- Figs. 80, 89 and 98. Cysts of *E. nana*.
- Figs. 81, 90 and 99. *Dientamoeba fragilis*.



THE OCCURRENCE OF HELMINTHS AND COCCIDIA IN PARTRIDGES AND PHEASANTS IN DENMARK

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An investigation of the food and parasites of game birds was initiated in October 1936 under the direction of Prof. M. Christiansen of the State Veterinary Serum Laboratory and Prof. R. Spärck of the University of Copenhagen. This investigation was under the auspices of the Jagtfond (Game Foundation) of the Ministry of Agriculture.*

Since that time 143 adult and 56 partridge chicks (*Perdix perdix*) and 169 adult and 67 pheasant chicks (*Phasianus colchicus*) have been sent in for examination. They came from diverse localities and at different times of the year. Not all were examined for all parasites, since the methods of collecting were improved during the course of this study. The data concerning the numbers of each species of parasite are roughly quantitative. It is felt that they present an adequate preliminary picture both of the various parasites involved and the intensity of the infections therewith. Although the number of birds examined was rather small, several interesting points have been revealed concerning the variation in numbers of certain parasites in pheasants as compared with partridges and also the variation in the number of certain parasites attributable to age differences of the hosts.

MATERIALS AND METHODS

The helminths were collected under low magnification of a binocular microscope; the trachea, crop, and proventriculus being examined directly. For the crop this proved to be an unsatisfactory method. As a consequence *Capillaria contorta* was not observed until the latter part of the investigation when the crop was submerged in physiological salt solution. The contents of the small intestine and ceca were sieved through 1 and 0.5 mm wire mesh screens and all material which did not pass was washed into Petri dishes and the helminths removed and counted. The washings which passed through the sieves were centrifuged, the supernatant fluid was poured off, and the sediment resuspended in 50 per cent sugar solution and again centrifuged. Standard samples of the surface film were then taken with a platinum loop. Either coccidial oöcysts were counted in toto or 25 random fields were counted

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for calculation of total numbers. With caution these data may be utilized in estimating the density of infections.

RESULTS

Although an acanthocephalan, cestodes, trematodes, and nematodes were encountered during the course of this study, only the last were commonly occurring parasites. The others were of infrequent occurrence and infections were invariably light.

Acanthocephala

A single specimen of *Prosthorrhynchus transversus* (Rud., 1819) was recovered from an adult partridge. The parasite was also observed once in an adult pheasant. This acanthocephalan is a common parasite of various passerine birds but has been previously recorded as a rather rare parasite of pheasants and partridges in England by Clapham (1938).

Cestodes

Cestodes were found in nine pheasant chicks; no adults were so parasitized. Partridge chicks as well as adults were infected, although only in small numbers. For the most part the worms were in a bad state of preservation and impossible to identify. When they could be determined they were shown to be *Raillietina echinobothridia* (Megnin, 1880). Clapham (1935) reported this form in English pheasants and partridges.

Trematodes

Echinostomes were recovered from the intestine of two partridge chicks and also from two pheasant chicks. None were found in older birds. This appears to be the first report of echinostome parasites from pheasants. Clapham (1936) has reported *Echinostoma revolutum* from partridges. It is probable that the worms herein reported are this species.

Nematoda

Nematodes were by far the most common helminths encountered in this investigation, both as to the variety of species involved and the degree of parasitism observed. While some species were either of such rare occurrence or were present in such small numbers that they could not be viewed as seriously interfering with the well-being of the hosts, others must be looked upon as rather serious parasites, particularly in pheasant and partridge chicks as well as in young adults.

Syngamus trachea (Montagu, 1811). This nematode was comparatively rare in both partridges and pheasants. In the latter it was found occasionally in the chicks but only once in an adult bird. In partridges it was never observed in the chicks, although it was an occasional parasite in adult birds. Infections were light as a rule. This parasite is common in fowl in Denmark and has been incriminated by Christiansen (1935-36) as provoking the most important helminthic disease in pheasantries.

Apparently *S. trachea* is a more frequent as well as a more serious parasite of game birds in England than in Denmark, if one may judge from various reports (Clapham, 1935, and others).

Dispharynx spiralis (Molin, 1858). This parasite of the proven-triculus showed, like *Syngamus trachea*, differences in the frequencies with which it was found in pheasants and partridges. In the former it appeared only in chicks and the infections were light. In partridges on the contrary, it was of more common occurrence in both chicks and adults but was encountered most often and in the largest numbers in young adult birds. It is notable that the heaviest infections were concentrated in birds from one definite area, thus suggesting the existence of locally favorable factors for the parasite. This species has not previously been noted from partridges in Europe. It is curious that this relatively common parasite of game birds in Denmark has apparently not been encountered in the extensive investigations on the parasites of game birds in England. Cram (1931) has reported it from *P. perdix* in the United States and also from pheasants (1928).

Capillaria contorta (Creplin, 1839). According to Cram (1936) this parasite is known from a large number of birds including partridges and pheasants in which it occurs in the ingluvies and esophagus. As already noted, the direct examination of this portion of the digestive tract did not reveal the presence of this nematode during the first part of this investigation. The parasites were first observed when the infected tissues were submerged in physiological saline. They were only found in pheasants but were probably overlooked in partridges. Apparently they are common, for eight of 11 birds examined were infected. This parasite has not been reported from partridges or pheasants in England.

Capillaria longicollis (Rud., 1819). This nematode is a not infrequent parasite of both pheasants and partridges, occurring in about 25 per cent of those examined. The infections seemed to be slightly heavier in pheasants than in partridges and heaviest during the summer months.

Capillaria columbae (Rud., 1819). *C. columbae* is here reported from partridges for the first time. It was encountered on four occasions, in both chicks and older birds from widely separated areas at all times of the year in light infections. It was never found in pheasants.

Capillaria collaris (von Linstow, 1873). It is only with hesitation that these cecal parasites are referred to the species described by von Linstow. In most respects they agree very well, especially concerning the spicule and spicule sheath of the male. In the present female specimens there is a protruding membranous appendage of a form differing from that of *C. longicollis*. According to Morgan (1932) and Freitas and Almeida (1935) this is characteristic only of immature females of *C. collaris*. The same observation was made by Koffman (1939) who

distinguished *C. retusa* Railliet and *C. collaris*, although these species are considered synonymous by Freitas and Almeida (1935). Curiously Koffman reported that *C. collaris* was recovered from the small intestine instead of its normal habitat, the cecum.

There was a marked difference in the frequency with which this parasite occurs in adult partridges and pheasants, three per cent in the former, 75 per cent in the latter. In the pheasants, the density of infection was appreciable, whereas in partridges the infections were very light. Oddly, both the incidence and degree of infection with *C. collaris* was very low in pheasant chicks. This is the first report of *C. collaris* from pheasants.

Ascaridia compar (Schränk, 1790). While this nematode is commonly reported from partridges and a number of other gallinaceous game birds, it has never been reported from pheasants. In the present investigation it was encountered only twice from partridges. It is obviously not an important partridge parasite, although it is a frequently occurring nematode in game birds other than partridges and pheasants in Norway and Sweden. Possibly *A. compar* is, as suggested by Baylis (1936), a synonym of *Ascaridia galli*.

Heterakis gallinae (Gmelin, 1790). This nematode was the most commonly occurring helminth in chicks and adults of both hosts from all localities studied. The infections in chicks tended to be slightly heavier than in adults; the incidence of the parasite in pheasants was twice that observed in partridges, 81 and 40 per cent respectively. The infections in the former were ten times as heavy as those in the latter host.

Trichostrongylus tenuis (Mehlis, 1846). This important parasite was present in about 20 per cent of the adult partridges and pheasants examined although both the incidence and degree of infection seemed to vary with different localities. The heaviest infections were observed during the summer months, although no massive infections such as are reported for fatal cases of trichostrongylosis were ever encountered. It seems reasonable nonetheless to consider this parasite potentially dangerous for Danish pheasants and partridges. The wide general distribution of the parasite might lead, under favorable environmental circumstances, to a serious epizootic.

Coccidia

Altogether there were seven species of coccidia encountered, four in partridges and three in pheasants, although for present purposes they will be treated en masse. They were naturally of very common occurrence and were markedly more frequent in chicks and young birds than in older birds. As with most of the other parasites which have been enumerated, coccidia were more common in pheasants than in partridges and the infections were likewise heavier in the pheasant. Christiansen

(1935-36) has already indicated that coccidiosis is a well known cause of disease in both wild and pen-reared pheasant chicks in Denmark.

In Table 1, the frequency of infection of the two hosts with the various species of parasites is shown as a percentage for adult birds and chicks.

TABLE 1.—The frequency with which Danish partridges (143 adults and 56 chicks) and pheasants (169 adults, 67 chicks) were parasitized with various species of helminths and coccidia. The infections encountered are expressed as a percentage of the number of birds examined

	Partridges Per cent parasitized		Pheasants Per cent parasitized	
	Adults	Chicks	Adults	Chicks
Acanthocephala	1	0	1	0
Cestodes	2	7	0	13
Echinostomes	0	4	0	3
Nematoda				
<i>Syngamus trachea</i>	5	0	1	10
<i>Dispharynx spiralis</i>	9	6	0	3
<i>Ascaridia compar</i>	2	0	0	0
<i>Capillaria longicollis</i>	20	26	23	28
<i>C. contorta</i>	..	..	73*	..
<i>C. columbae</i>	4	2	0	0
<i>C. collaris</i>	3	2	75	6
<i>H. gallinae</i>	40	52	81	74
<i>Trichostrongylus tenuis</i>	24	2	22	6
Coccidia	48	62	82	73
Not parasitized	20	13	2	24

* Only 11 birds examined.

DISCUSSION

Although much of the information which has been gained from this study of the parasitic fauna of Danish pheasants and partridges cannot be considered as new, a knowledge of the variety of parasites involved as well as information on their relative frequency and parasitic density was a necessary prerequisite for further work. Granting that parasites may condition to some extent game management and control problems, the present investigation, even though based on the examination of a comparatively small number of birds, provides a basic quantitative background not previously available for Danish game birds.

The indications that the relative importance of certain parasites may here differ considerably from those prevailing in other game areas, as for example in England, seem particularly important. Interesting also is the fact that while the parasites tabulated in Table 1 are for the most part common to both pheasants and partridges, the frequency of their occurrence in some instances indicated a variation in the apparent favorableness of these hosts for these specific parasites. Thus *Dispharynx spiralis* occurred more commonly in partridges than in pheasants, whereas *Capillaria collaris*, *Heterakis gallinae*, and coccidia occurred more often in pheasants. Others, such as *Ascaridia compar* and *Capillaria columbae*, occurred exclusively in one host; still others, such as *C. longicollis* and *T. tenuis* occurred with about equal frequency in both hosts.

The most pronounced difference attributable to age of the host occurred in the case of *Trichostrongylus tenuis* in both pheasants and partridges, the chicks being both infrequently and lightly infected in comparison with the adult birds. On the other hand, in the case of the echinostomes and cestodes, the infections were almost always in chicks and rarely in adults. Age differences with other parasites were suspected, but the limited data do not justify definitive statements.

In conclusion it may be observed that the data offer a glimpse of a complex biological and ecological relationship which must certainly vary from season to season and contain elements which need to be more fully understood in order to ensure maximum numbers of these game birds over their range. In some places game keeping is practiced intensively

SUMMARY

A total of 143 adult and 56 partridge chicks (*Perdix perdix*) and 100 adult and 67 pheasant chicks (*Phasianus colchicus*) from various game keeping areas in Denmark have been examined for internal parasites. In addition to coccidia, there were found an echinostome, cestodes, an acanthocephalan and nine species of nematodes. The echinostome, apparently *Echinostoma revolutum*, has not heretofore been reported from pheasants. The appearance of *Dispharynx spiculis* in partridges seems to be a new European host record. *Capillaria columbae* from partridges and *C. collaris* from pheasants are new host records for these parasites.

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STUDIES ON THE LIFE HISTORY OF *EUPARYPHIUM* *MELIS* (TREMATODA: ECHINOSTOMIDAE)¹

PAUL C. BEAVER

On August 8, 1938, an undescribed species of echinostome cercaria was found in one of 133 adult *Stagnicola emarginata angulata* collected from North Fishtail Bay on Douglas Lake, Michigan. Its behavior and morphology were recorded and certain facts concerning the metacercaria were obtained. On August 10 of the following summer, another snail infected with this species was discovered. On this occasion 550 *S. emarginata angulata* were taken from near the first location at Nutting Bay on Douglas Lake. From experiments on the material thus obtained, the redia, cercaria, metacercaria, and adult stages were determined for a species which seems to be identical with *Euparyphium melis* (Schrank, 1788).

THE CERCARIA

(Figs. 7, 8, 9)

The cercariae emerge between 5 and 9 PM, and live about 24 hours in the laboratory. They swim and creep with comparatively great speed, and characteristically are nearly equally distributed throughout the container until they become spent and settle to the bottom. During the period soon after emergence, they alternately creep and swim in intervals of 2 to 3 minutes. In behavior and general appearance, this cercaria is strikingly similar to the cercaria of *Psilostomum ondatrae* Price (Beaver, 1939b). Contracted, the body is somewhat angular in outline with the width and length about equal; extreme extension brings its width to less than one-eighth its length. The tail may contract to one-third its greatest length. The cercariae readily detach the tail when put under pressure of the coverglass and are crushed more easily than most echinostomes.

Description: Body relatively transparent, colorless; elongate but with stout proportions, the post-acetabular region slightly set-off from the remainder; greatest width in relaxed condition near middle of pre-acetabular region. Cuticula thick but relatively soft, bearing extremely minute spines anteriorly, and extending beyond margins of suckers to form a transparent ruffle on the border of each. Collar spines easily seen, distributed as follows: 4 angle spines, 6 laterals in a single row, and 7 dorsals in a double row, 4 of the dorsals being oral. Angle spines much larger than

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the others. Although the spines are well developed the collar itself is almost indistinguishable.

Oral sucker terminal, or slightly subterminal, muscular. Width of pharynx one-third that of oral sucker. Acetabulum in posterior third of body, very muscular, slightly protrusible, somewhat larger than oral sucker. Gut without lumen but evident in both living and fixed preparations. Excretory bladder with large posterior chamber and smaller anterior one, both contractile. Excretory siphons arise mesially from bladder, extend much coiled to sides of acetabulum where they describe a sharp lateral loop, then become inflated as they form three (sometimes 4) rounded loops containing 15 to 25 large scattered granules along sides of esophagus. Before reaching level of pharynx siphons become small and straight; at level of angle spines they reverse their course and extend unbranched to extreme posterior end of body where branching occurs. Vibratile tufts in the latter are about 10 in number. Flame cells 17 or 18 on each side, apparently arranged in 6 groups of 3 each, half above and half below the acetabulum. The 3 in the region of the oral sucker are definitely arranged in a single group, the others appear to be so arranged.

Portion of body above level of pharynx very clear; remainder of body more granular due to angular masses of finely granular cystogenous material. Six pairs of large nuclei appear along sides of esophagus in stained specimens. No cephalic ducts were observed. Tail terminal, plain, having no fins or folds, and provided with both longitudinal and circular muscles to the extreme tip. Caudal excretory tube extends through first fourth of tail, bifurcates, and attaches to, but does not empty through, the lateral wall.

Measurements on fixed material extended as in Fig. 7 are: body length, 0.384 mm; tail length, 0.545 mm; body width at crown, esophagus, and acetabulum respectively, 0.08 mm, 0.18 mm, 0.15 mm; greatest width of tail, 0.052 mm; oral sucker, 0.060 by 0.054 mm; pharynx, 0.024 by 0.018 mm; esophagus, 0.169 by 0.012 mm; acetabulum, 0.054 by 0.078 mm; angle spines, 18–20 μ by 2–3 μ ; dorsal spines, 12–14 μ by 2–3 μ . The following additional measurements were made on living material under a coverglass: diameter of siphon granules, 4–14 μ ; greatest extension of body about 0.5 mm; tail, 0.55 by 0.06 mm; acetabulum, 0.09 by 0.08 mm; oral sucker, 0.08 by 0.07 mm; length of flame cells, 2 μ .

THE REDIA

(Figs. 10, 11)

The infections were old and both of the snails examined had been in the laboratory more than three weeks. They were alive when opened, however, and although only a few cercariae were emerging during the period immediately preceding the examination, an abundance of active rediae of various sizes were obtained from the liver and gonad. The long period in the laboratory may account for the presence of not more than 3 mature cercariae in the largest rediae.

Description: Living specimens very active, especially younger ones; body long, slender, sharply tapered at posterior end; colorless even in oldest individuals. Appendages unusually long and motile, attachment distinctly ventral; distance from posterior end to appendages about one-fourth total body length but varies between one-fifth and three-fifths. Collar well developed, motile, conspicuous in all states of contraction and after fixation; completely divided into 4 lobes, a dorsal, a ventral, and 2 laterals; located well back of anterior end, half-way between oral sucker and birth-pore. Birth-pore dorsal and usually very conspicuous. Sucker ordinary. Gut large, often quite filling the body, and extending well beyond appendages. Measurements on well extended fixed material: average mature specimens, 1.4–1.7 mm in length; sucker, 50 by 50 μ to 80 by 60 μ . Excretory bladders somewhat nearer appendages than collar; two groups of flame cells on either side, each containing 24 or

more flame cells in specimens around 1.5 mm in length; anterior pair located just posterior to collar, the other one at base of appendages. No mother rediae were observed although immature rediae that apparently had recently emerged were numerous. The smallest of these was 0.32 mm in length.

THE METACERCARIA

(Figs. 12, 13)

Under pressure of the coverglass cercariae often lose the tail and form imperfect cysts. Normal encystment could not be induced, however. When isolated with various species of fish (*Ameiurus nebulosus*, *Perca flavescens*, *Umbra limi*, *Lepomis pallidus*, *Ambloplites rupestris*, and *Percina caprodes*) some of the cercariae would spend short periods creeping on them and a small percentage would encyst in the nostrils and cloaca. This was observed more often in *Ameiurus nebulosus* than any other species of fish.

When isolated with tadpoles, the cercariae behave in a very different manner. They are quick to settle on the tadpoles, begin rapid creeping immediately, and enter the cloaca in a remarkably short period. Their behavior in this respect is identical with that previously described for *Echinostoma revolutum* (Beaver, 1937) in which the direction taken is ventro-posterior towards either the cleft between the body and tail or the ventral margin of the tail, and from there directly to the cloaca. Now instead of following the mesonephric ducts forward as is the case in *Echinostoma revolutum*, *Echinoparyphium recurvatum*, *Nephrostomum ramosum*, and some other species of echinostomes, they penetrate the cloacal wall, migrate a short distance through the tissues, and encyst either in the skin or subcutaneous tissues. Occasionally a cyst is found in the deeper connective tissues. The cyst is almost spherical and extremely thin-walled when formed. The wall remains thin in cysts located in the epidermis, but in the dermis and deeper tissues a heavy fibrous wall of host tissue is added in 5 or 6 days. Heavily infected tadpoles are readily recognized by local swelling and rough thick skin in the cloacal region (Fig. 13). Exclusive of host tissue, the cysts vary from 0.152 by 0.145 mm to 0.170 by 0.170 mm. The true cyst-wall is 2–3 μ thick and the host tissue after 37 days was found to be 0.120–0.133 mm thick on cysts that were removed from the host.

During the first three weeks in the cyst there is continuous growth in the metacercaria, and noticeable changes in its morphology occur. As it increases in size the body becomes flexed ventrally, and the suckers and pharynx, especially the latter, become proportionately larger than in the cercaria; the gut develops a lumen; the siphon granules remain the same in number and size but extend into the post-acetabular region; the cuticular spines become relatively large and conspicuous; and the collar itself becomes more pronounced. The following measurements were made

(without pressure) on a living specimen 21 days old: diameter of acetabulum, $90\ \mu$; oral sucker, 70 by $60\ \mu$; pharynx, 36 by $30\ \mu$; length of dorsal collar spines, 18 – $20\ \mu$; length of angle spines, 2 – $28\ \mu$; body extended, 0.42 by 0.16 mm.

The metacercaria is probably infective after three weeks. At any rate, there is no further noticeable development in stages up to 37 days of age.

THE ADULT

(Figs. 1–6)

Metacercariae under 6 days of age were fed to a rat, 2 mice, a cat, and a pigeon. All were negative when examined 3 and 8 days after feeding. It was later observed that the metacercariae were undergoing development in the cysts and because experimental material was relatively scarce, no further attempts were made to obtain adult worms until the metacercariae were 3 weeks old. Then 2 kittens and a ferret were fed infected tadpoles. The kittens were each given 2 tadpoles carrying about 50 cysts. Both were negative when examined after 5 and 8 days. The ferret was given 5 tadpoles carrying about 100 metacercariae, 26 to 37 days of age. It was fed nothing but fresh pork liver and canned fish. A severe diarrhea developed on the 9th day and persisted through the 10th and 11th days. Because unhealthy animals frequently lose intestinal worms, and especially because it was observed that the ferret had eaten about 30 square inches of a cotton towel, it was thought advisable to terminate the experiment. One immature worm was found high in the duodenum. About 2 hours later, after this worm had been studied, described, and fixed, the stomach was examined. Most of the toweling was still in the stomach. No worms were found.

The tadpoles used in these experiments were collected from an acid lake where snails do not occur, and where many other tadpoles have consistently been found by numerous investigators to be negative for larval trematodes. There seems to be no doubt about the history of the worm found in the ferret. The cephalic spination is identical in the cercaria, metacercaria, and adult. The character of the suckers, cuticula, and body proportions are likewise similar in the metacercaria and adult.

Because the experimental worm is immature, some of the taxonomically important features of the echinostomes, such as vitellaria, ova, and cirrus sac complex, can not be described for it (Fig. 6). Other structures, however, are well formed and enough of its specific characters are evident to make its identification positive.

Description: Body fusiform, rather stout in moderate state of contraction. Length, 1.46 mm; width at acetabulum, 0.348 mm; width of collar, 0.18 mm. Oral sucker sub-terminal, 0.09 by 0.84 mm; pre-pharynx short; pharynx, 0.070 by 0.056 mm; esophagus and ceca simple, the latter reaching almost to posterior end. Acetabulum powerful, situated above middle of body, 0.204 by 0.210 mm. Testes median,

half way from acetabulum to posterior end, round. Ovary immediately anterior to testes, slightly dextral, round. Uterus short, coiled transversely. Cirrus sac pyriform, widest anteriorly, dorsal to and in front of the anterior part of acetabulum. Cuticula thickly set with minute spines on anterior half of ventral side and on pre-acetabular portion of dorsal side. Collar not pronounced but clearly evident; spines arranged as in cercaria and metacercaria; angle spines about 37 by 6 μ , dorsal spines about 32 by 5 μ . Excretory pore distinctly dorsal.

There are two known species that closely resemble the experimental worm. *Euparyphium melis* (Schränk, 1788) is found in several species of European MUSTELIDAE (*Mustela foina*, *Lutra vulgaris*, *Meles taxus*) (Dietz, 1910) and also has been reported from the mink (*Mustela vison*) in North America (Law and Kennedy, 1932). *Euparyphium inerme* (Fuhrmann, 1904) occurs in the otter (*Lutra* sp.) in Java, and has been reported once from North American mink (Price, 1932). Other species which have been placed in the genus *Euparyphium* are: *E. jassyense* Leon and Ciurea, 1922, *E. capitaneum* Dietz, 1909, *E. incrassatum* (Dies.), *E. suinum* Ciurea, *E. spiculator* Obitz, 1933, *E. guerreroi* Tubangui, 1931, *E. ilocanum* (Garrison, 1908), *E. murinum* Tubangui, 1931, and *E. malayanum* (Leiper, 1911).

Through the kindness of other collectors, several good series of worms were received from the mink and otter in this country and from the otter in Java. The material from Java corresponds in nearly all details with the description of *Euparyphium inerme*. Cuticular spines were absent in Fuhrmann's specimens and spines of the cirrus were not mentioned in the description. Both are present in my specimens and they all have 27 instead of 26 collar spines. In spite of these differences in observations, it seems probable that the two collections are of the same species. Neither the experimental worm nor any of the series from North American hosts can be *E. inerme*. In the latter, the testes are extremely long and winding in older worms, and are very elongate in immature ones. The collar spines are longer and more slender, the acetabulum is relatively larger, and the body is more compressed dorso-ventrally in *E. inerme*.

The material from this country is without question the same species as that reported from the mink by Law and Kennedy (1932) and described as *Euparyphium melis* (Schränk). Comparison of the European form (based on Dietz, 1910) with the North American specimens reveals a few possible points of difference. In the former, the 27 collar spines are rounded at both ends and 10 of them are described as oral. The accompanying text figure shows but 4 distinctly oral, however. In well fixed specimens of my collection, all spines of the collar are pointed at the outer ends and 4 of the dorsals are oral in position. That these differences are not real, but rather are due to different post-mortem histories and different interpretations of the structures observed, can not be demonstrated now. The two collections are so similar in other respects that a new name

for the American species probably would be rejected as soon as the European form is better known.

Since it is not certain that the European and American forms are identical, and no taxonomic description has been published for the latter, such a description is here given, based on whole mounts and sectioned specimens from the mink collected in Michigan and Minnesota, and from the otter collected in Michigan.

Euparyphium melis (Schrank)

Description: Body elongate, rounded at both ends; sides tapering anterior to acetabulum, more parallel in hind body. Mature worms range between 3.86 by 0.65 mm and 10.5 by 2.1 mm, with the greatest width at or just back of the acetabulum. Cuticular spines very minute, extending to acetabulum dorsally and to testes ventrally. Oral sucker sub-terminal, walls thick, shape pyriform with narrowest part posterior; its posterior border reaching level of angle spines. Crown moderately developed, well set off in living worms and well fixed ones, but much less so in worms fixed post-mortem. Collar spines large, rather stout, abruptly tapered at outer end, widest level often just proximal to taper; 27 in number with 4 on each angle, 6 in a single row on each side, 4 orals and 3 aborals on the dorsal side. Angle spines in 2 pairs, one over the other, the deeper or more posterior pair being somewhat larger than the other. Lateral spines about equal, and generally much smaller than angle spines. Dorsals slightly larger than laterals but smaller than those of the angles; orals slightly larger than aborals. Pharynx ovoid, length about two-thirds diameter of oral sucker. Neck narrower than crown in extended worms, broader in contracted ones. Bifurcation of esophagus immediately in front of acetabulum. Wall of esophagus same as in ceca. Acetabulum almost spherical, with thick walls; depth usually less than diameter; located far above middle of body.

Testes tandem, usually contiguous; tend to be plain and round, become elongate in older worms and may have 1 to 4 shallow notches in the margins; their location varies with length of worm but tends to be nearer acetabulum than posterior end. Cirrus sac elongate, extending just beyond middle of acetabulum in some specimens. Seminal vesicle long, slender, straight; divided into large posterior and smaller anterior chambers; wall of anterior portion thick, containing 30 to 36 muscle bands which are very prominent in transverse sections. Pars prostatica about same length as muscular portion of seminal vesicle; prostate gland moderately developed. Cirrus very long, much coiled when withdrawn; length about 0.8 mm in specimens 5-6 mm long; spines on cirrus thickly set, about 2μ long; evident in sections or when extended, and may be seen inside of cirrus sac in some specimens.

Ovary regular in outline, spherical to pyriform, located on right side and slightly ventral. Oviduct arises from dorsal side. Laurer's canal well developed with large lumen and median dorsal opening to outside. Mehlis' gland broadly oval with sharp regular margins; compact cells of two distinct types, larger basophil proximal cells and more numerous but smaller neutrophil cells. The most proximal portion of uterus forms seminis receptaculum uterinum; true seminal receptacle absent (Fig. 5). Uterus short, forming 3 to 5 transverse coils back of acetabulum. Metraterm on left side of cirrus, not especially muscular and not well set off from uterus; opening of metraterm back of cirrus. Vitellaria extend from level of anterior margin of ovary to posterior end of body, the two fields confluent along dorsal side and to a less extent along ventral side back of testes; often there are almost no follicles along mid-ventral side. Vitelline follicles irregular in shape, 40-120 μ in diameter. Vitelline ducts pass ventral to ceca and enter vitelline reservoir separately; reservoir well developed, situated dorsal and posterior to remainder of female complex. Ova large, with heavy shells, amber in color; 117 by 72 μ to 130 by 84 μ . Excretory pore sub-terminal, distinctly dorsal.

Measurements on average specimen: length 6.1 mm; width at acetabulum, 0.82 mm; width of collar, 0.52 mm; oral sucker, 0.282 by 0.291 mm; pharynx, 0.198 by 0.148 mm; distance between suckers, 0.68 mm; diameter of acetabulum, 0.214 mm; cirrus sac, 0.602 by 0.260 mm; distance between acetabulum and ovary, 0.906 mm; ovary, 0.256 by 0.215 mm; Mehlis' gland, 0.900 by 0.215 mm; anterior testis, 0.563 by 0.382 mm; posterior testis, 0.700 by 0.420 mm; length of post-testicular region, 2.75 mm; ventral angle spines, 100 by 32 μ ; dorsal angle spines, 120 by 38 μ ; third lateral spine, 80 by 26 μ ; second oral dorsal spine, 104 by 32 μ ; middle aboral spine, 98 by 28 μ ; body spines at level of genital pore, 18 μ .

Hosts: *Mustela vison* and *Lutra canadensis*.

Location: Stomach and upper duodenum.

Distribution in North America: Michigan, Minnesota, Ontario.

Museum specimens: U. S. Nat. Mus. Helm. Coll. Nos. 36686-36691.

Autopsy records give both the stomach and duodenum as sites of infection. The experimental worm reported here was taken from the duodenum near the pylorus. None was found in the stomach although several may have been present and overlooked because of the unfavorable conditions under which the examination was made.

DISCUSSION

The life cycle of *Euparyphium melis* differs markedly from all life cycles yet determined for apparently closely related species. *Euparyphium ilocanum* as determined by Tubangui and Pasco (1933) differs by having a redia with a complete collar and short gut, a cercaria with narrow well-filled siphons and pronounced collar, a metacercaria that occurs in snails in heavy-walled cysts, and an adult with very numerous collar spines, long uterus, and vitellaria reaching far above the ovary. *Euparyphium murinum* as determined by Tubangui (1932) differs in exactly the same characters as *Euparyphium ilocanum*, having only 45 to 46 collar spines instead of 51, however. Rao (1933) did feeding experiments with metacercariae in snails and fish from which he obtained adult echinostomes in two dogs and a kitten. He regarded these adult worms as identical with *Euparyphium malayanum* (Leiper, 1911). Since both snails and fish were fed to the experimental animals, the origin of the worms is uncertain.

The four-lobed or broken collar of the redia in *E. melis* is the third of its kind to be described within the past year. The other two are *Psilostomum ondatrae* and *Pectasiger nitidus* (Beaver, 1939a, 1939b). Since many other echinostome and psilostome rediae have been described as having a complete collar, it was thought good to reexamine the collar of all forms available. It was found that the rediae of *Echinostoma revolutum*, *Echinoparyphium recurvatum*, *Stephanoprora* sp., *Echinochasmus* sp., a 35-spined echinostome (apparently *Cercaria limbijera* Seifert), and *Hypodaeium* sp. have the simple complete collar.

The purpose of the feeding experiments primarily was to connect the larval stages with an adult species. Sufficient numbers of cercariae for

other important experiments were not available. The miracidium is undescribed, the length of time necessary for metacercarial development is not known exactly, and the usual location of the adult stage within the host has not been determined satisfactorily.

SUMMARY

1. A new echinostome cercaria having 27 collar spines and plain tail is described from *Stagnicola emarginata angulata* from the region of Douglas Lake, Michigan.

2. The redia is of the long gut type, having prominent appendages, broken collar, and no pigment.

3. Tadpoles of several species were experimentally infected with the metacercaria which forms a thin-walled cyst in the skin or subcutaneous tissues in the region of the cloaca.

4. The adult stage was obtained by feeding metacercariae which were over three weeks of age to a ferret.

5. The adult worm is identical with a species previously reported from the North American mink (*Mustela vison*), and probably is identical with the European species, *Euparyphium melis* (Schrank, 1788).

6. The North American form is redescribed.

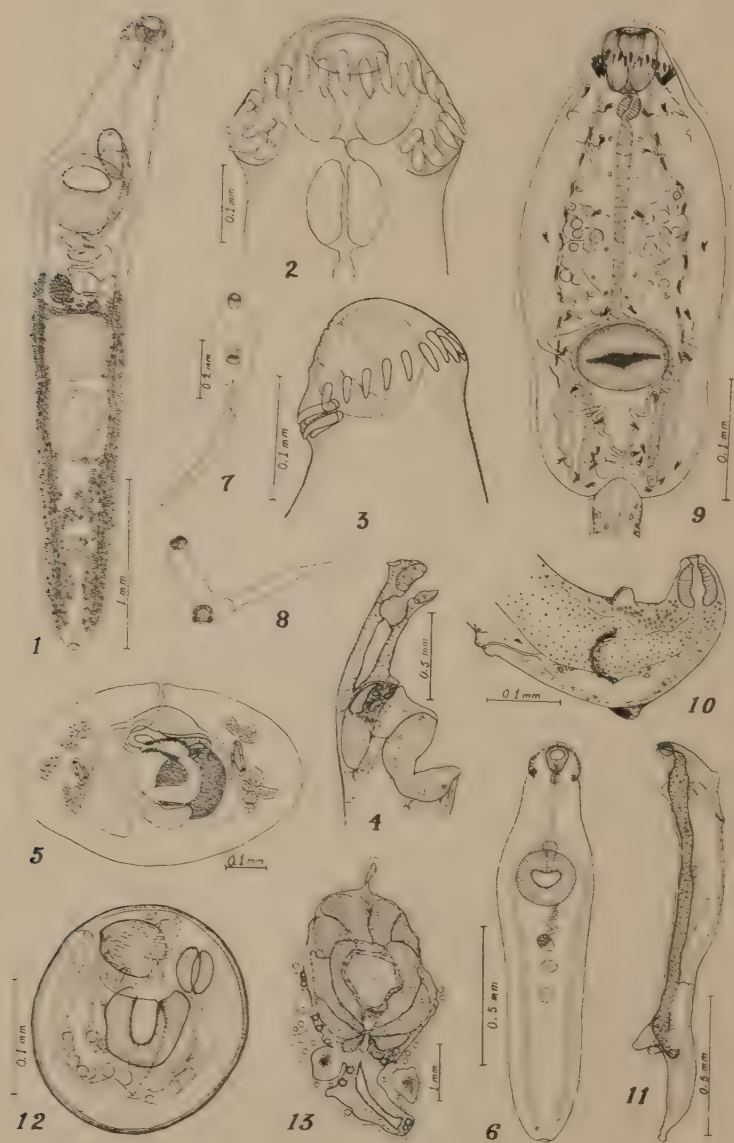
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EXPLANATION OF PLATE, p. 44

Drawings made with the aid of a camera lucida

- FIG. 1. Adult of average size from a mink. Ventral view.
FIG. 2. Head crown of the specimen shown in Fig. 1.
FIG. 3. Head crown of the specimen shown in Fig. 6. Lateral view.
FIG. 4. Sagittal section through the suckers, cirrus sac, genital pore, esophagus, and pharynx of an adult.
FIG. 5. Graphic reconstruction of the female genital complex viewed from the posterior end. The dorsal side is above.
FIG. 6. Young adult from an experimental ferret. Ventral view.
FIGS. 7 and 8. Cercariae showing typical attitudes of the body and tail.
FIG. 9. Cercaria. Ventral view of the body moderately extended. (Outlines drawn from an unflattened fixed specimen. Details of the excretory system added.)
FIG. 10. Redia. Lateral view of the anterior end showing relationship of the mouth, gut, collar, and birthpore.
FIG. 11. Redia. Lateral view of an average specimen.
FIG. 12. Metacercaria in cyst. Age 17 to 21 days.
FIG. 13. Transverse section of an infected tadpole, cut at the level of the cloaca and posterior limb buds. Location and size of metacercariae are shown by the small open circles. About 30 metacercariae appear in this section.

*Euparyphium melis.*

TRYPANOSOMIASIS IN THE FLORIDA COTTON RAT, *SIGMODON HISPIDUS LITTORALIS*

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The blood of a number of wild Florida cotton rats (*Sigmodon hispidus littoralis*)¹ recently received for experimental purposes by investigators in this laboratory, was examined for the presence of natural infections with trypanosomes, and some of the animals were found to be positive.² Since, so far as the writer is aware, no natural trypanosome has previously been described for this species of host, the natural and experimental infection with this form has been studied carefully in some detail. At the same time, the susceptibility of the cotton rat to induced infection with other species of trypanosomes has also been investigated. The observations made during the study provide the data for this report.

THE NATURAL TRYPANOSOME OF THE FLORIDA COTTON RAT

Incidence of infection. The tail blood of 90 cotton rats was examined for trypanosomes promptly on the arrival of these animals by express from the commercial shipper (Zoological Research Supply, Englewood, Florida), who had obtained the animals from the field. Of this number, 18 (20 per cent) were naturally infected with the trypanosome, the parasite being found in the tail blood. The host animals weighed from 50 to 150 grams, small and large animals having about equal incidence of infection. The animals appeared not to suffer from the trypanosome, none succumbing from the effects of either the natural infection or the experimentally induced infections.

Description of the trypanosome. In fresh blood examined ($\times 430$) under the cover slip, the trypanosomes moved actively, but made comparatively little headway. They did not readily leave a given microscope field, and often confined their activity to a small zone of the blood fluid from which by their movements they had pushed back the erythrocytes. In blood films treated with Wright's stain, the forms appeared as typical trypanosomes, with a nucleus set only very slightly, if at all, forward of the cell center. A well-developed flagellum and undulating membrane, and a fairly prominent kinetoplast were present. The posterior end of the cell was sharply pointed. No multiplying cells were observed in films of the peripheral blood, although films were prepared only from

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cotton rats infected ten days or more previously. Measurements of 25 trypanosomes with a filar ocular micrometer revealed the following dimensions: length range $14.8\ \mu$ to $20.0\ \mu$ with mean length of $16.9\ \mu$, and greatest breadth range $1.6\ \mu$ to $2.5\ \mu$, with a mean greatest breadth of $2.0\ \mu$. These dimensions for length exclude the free flagellum which ranged from $2\ \mu$ to $5\ \mu$, and those for greatest breadth exclude the undulating membrane. The blood films used for these measurements were prepared by the following technic: They were let dry without special fixation; Wright's stain was applied for 1 minute; an equal amount of tap water was added to the Wright's stain and the mixture let stand on the film for 3 minutes; and the film was washed in tap water. After drying, the film was examined with an oil immersion objective ($\times 93$) and the filar micrometer ocular ($\times 8$).

As yet, only the heart has been examined in section for fixed-tissue stages of the parasite. None was seen in its tissues.

Cultivation. Several attempts to cultivate the cotton rat trypanosome in N.N.N. medium were made, but with no success. Inoculated parasites survived up to four days in the test tube, but no evidence of their multiplication was noted. The addition of 0.1 cc of infected cotton rat blood to 5 cc of nutrient broth (a procedure the author has found successful for cultivating *T. cruzi* from the albino rat) also failed to result in the cultivation of the cotton rat trypanosome.

Normal course of an experimentally induced infection in the cotton rat. The natural trypanosome infection in the cotton rat can with facility be carried artificially by transfer of infected blood to other cotton rats. In the present work, blood from a heavily infected donor animal was emulsified in nutrient broth and inoculated intraperitoneally. Infections thus induced with this trypanosome generally remained patent for at least one month, following a prepatent period of 6 to 10 days. The peak of the infection occurred about one week after the parasites first appeared in the blood, the parasite count at this time occasionally reaching 25,000 organisms per cmm of tail blood. The data for the course of infection in a representative animal are given in Table 1.

Natural transmission. The natural vector of this trypanosome among

TABLE 1.—*The course of an experimentally induced infection with the cotton rat trypanosome in the cotton rat*

Cotton Rat No.	Parasite counts on designated days after infection											
	8	10	12	15	17	20	23	25	28	30	33	35
1	+	+	2T	8T	12T	9T	9T	7T	2T	++	+	0

Key to parasite counts:

0 = no parasites in 50 microscope fields ($\times 430$);

± = less than 1 parasite per field;

2± = from 1 to 5 parasites per field;

2T, 8T = thousands of parasites per cmm of tail blood by hemocytometer.

etc

cotton rats has not been determined. In the pelage of the animals, fleas and lice are often observed for a few days after the cotton rats arrive, but none of a number of such insects on dissection has been found infected with stages of trypanosomes. Mites as well as the fleas have been found in the litter of the shipping cages. The species of the insects and the mite have not been established.

Experimental inoculation of other hosts. Rabbits, guinea pigs, and albino rats and mice were inoculated with a broth suspension of the cotton rat trypanosome, to determine the susceptibility of these host species to the parasite. Of the several hosts only the albino rat—and of the rats only young individuals—proved susceptible to demonstrable infection. The susceptible albino rats were nurslings 9 days old when injected. They were positive on examination of tail blood after from 4 to 8 days, and occasional trypanosomes were seen thereafter for about 10 days. The infections were extraordinarily mild in every case, never as many as 1 parasite being seen per 10 microscope fields ($\times 430$). No infection resulted in a second group of nursing rats to which blood from these infected nurslings was transferred (see Table 3).

Effect of germanin and of normal human serum. The cotton rat trypanosome was tested for susceptibility to germanin (Bayer 205) and to fresh normal human serum. These materials are without effect upon many species of trypanosomes, including those of the *levisi* group, although they are highly active in destroying certain of the pathogenic trypanosome, such as *T. equiperdum* and its relatives. The present experiment was carried out upon 5 cotton rats already well infected with their natural trypanosome, to determine whether the cotton rat trypanosome resembled *T. levisi* or *T. equiperdum* in its susceptibility to germanin and human serum. To 2 of these, 10 mg of germanin was given intraperitoneally and, to a third, 2 cc of fresh normal human serum was injected by the same route. The trypanocidal activity upon *T. equiperdum* of both the drug and the serum was proved by a control test in albino rats infected with *T. equiperdum*. All five of the cotton rats were examined just prior to treatment and again 24, 48, and 72 hours after treatment. In no case was the number of trypanosomes in the cotton rats affected by the treatment, although both substances removed *T. equiperdum* from the peripheral blood of the albino rats within 24 hours.

STUDIES ON IMMUNITY WITH THE COTTON RAT TRYPANOSOME

Resistance to reinfection. A cotton rat which had recovered just two weeks earlier from a heavy infection with the cotton rat trypanosome (Rat No. 1, Table 1) was, along with a normal cotton rat,² reinoculated

² Since only field-caught animals were available for this work, one was never certain that new animals used as controls had not recovered from infection prior to arrival in the laboratory. This difficulty was overcome only by using a disproportionately large number of control animals in each experiment.

with the same trypanosome. The recovered cotton rat showed no infection at any time after reinoculation. The control cotton rat showed the parasite on the tenth day after infection, and the parasite count rose subsequently, with its peak about one week later. The results of this test are given, together with the results of a test for the passive transfer of immunity against this parasite, in Table 2.

TABLE 2.—*Specific immunity of the cotton rat after recovery from infection with the cotton rat trypanosome, and the transfer of this immunity in the serum*

Cotton Rat No.	Treatment	Parasite counts on designated days after infection					
		8	10	12	15	17	20
1*	Actively immune by recovery	0	0	0	0	0	0
2†	Passively immune	0	0	0	0	0	0
3	Normal control	0	+	2+	6T	7T	7T

Key to parasite counts: same as Table 1.

* Same as cotton rat No. 1, Table 1; reinoculated 2 weeks after recovery from initial infection.

† Immunized passively with 1 cc of serum from recovered cotton rat No. 1.

Passive transfer of acquired immunity in the serum. A very small normal cotton rat was inoculated together with the 2 cotton rats mentioned in the preceding section with a suspension of cotton rat trypanosomes. This animal was injected at the same time with 1 cc of serum from the cotton rat (No. 1) known to have recovered 2 weeks earlier from infection with the cotton rat trypanosome. The test animal given the specific antiserum remained permanently free of the parasites, although the control animal showed parasites on the tenth day subsequently. The parasite count in the control animal rose thereafter in the usual manner of this infection. It is indicated by this work, the results of which are shown in Table 2, that the immunity of the specifically recovered cotton rat can be passively transferred in the serum to a normal animal.

Thirty-five days after the initial injection of trypanosomes into the passively immunized rat, this animal was reinjected with the cotton rat trypanosome. After a prepatent period of 11 days, the animal became infected and showed the usual course of infection of a normal animal. It is thus shown that the passive immunity in the cotton rat against the cotton rat trypanosome is of the expected comparatively brief duration.

*Immunity of albino rats to *T. lewisi* and of cotton rats to their trypanosome after inoculation with the alternate parasite.* One normal cotton rat was injected with *T. lewisi* and three nursing albino rats were injected with the cotton rat trypanosome. No parasites were seen at any time during 17 days after inoculation in the cotton rat, although a control albino rat injected with the same suspension of *T. lewisi* promptly contracted a heavy infection. This cotton rat was then inoculated with the cotton rat trypanosome. The ensuing infection, which was first

patent twelve days later, lasted 30 days, although its intensity was extraordinarily low and on some days no infection was demonstrable. The three albino rats suffered very mild infections with the cotton rat trypanosomes (as stated also in an earlier section of this paper), and overcame their infections with no difficulty. On inoculation of *T. lewisi* to these albino rats three weeks later, no infection resulted, although control albino rats promptly became infected. From this, it is indicated, as shown in Table 3, that prior inoculation of the albino rat with the cotton

TABLE 3.—*Demonstration of (1) immunity of albino rat to T. lewisi after inoculation with cotton rat trypanosome, and (2) partial immunity of cotton rat to its natural trypanosome after inoculation with T. lewisi*

PART 1: ALBINO RATS

Animal No.	Course of cotton rat trypanosome infection, on designated days							Course of <i>T. lewisi</i> infection, on designated days			
	4	6	8	10	12	14	16	4	6	8	10
1	0	0	+	+	+	+	0	0	0	0	0
2	0	0	+	0	0	0	0	0	0	0	0
3	+	+	0	+	+	+	0	0	0	0	0
4	Controls, not infected with cotton rat trypanosome							3+	4+	4+	3+
5								3+	4+	4+	3+
6								2+	3+	+	0

PART 2: COTTON RATS

Animal No.	Treatment	Course of infection with cotton rat trypanosome, on designated days													
		8	9	10	12	15	17	21	25	27	30	33	35	36	
10	<i>T. lewisi</i> previously inoculated*	0	0	0	+	++	+	+	0	+	+	0	0	0	
11	Control	+	+	6T	15T	23T	13T	11T	7T	7T	8T	2+	+	0	

* Infection with *T. lewisi* in cotton rat No. 10 was never demonstrated directly in the peripheral blood.

† The heaviest infection with the cotton rat trypanosome occurred on the fifteenth day, when 22 parasites were seen in 50 microscope fields.

Key to counts of cotton rat trypanosome, same as Table 1.

Key to counts of *T. lewisi*:

0 = no parasites in 50 fields of microscope ($\times 430$);

+= less than 1 parasite per field;

2+ = between 1 and 5 parasites per field;

3+ = from 5 to 25 parasites per field;

4+ = over 25 parasites per field.

rat trypanosome, or of the cotton rat with *T. lewisi* protects these animals against infection from the subsequent inoculation of the alternate species of parasite. A close immunological relationship is thus indicated between these two species of parasites.

TAXONOMY OF THE COTTON RAT TRYPANOSOME

In a number of respects the cotton rat trypanosome appears to be distinct from *T. lewisi*: it does not grow easily, if at all, in the usual N.N.N. medium in which *T. lewisi* thrives; its nucleus is not so far forward in the cell as is that of *T. lewisi*; and it does not produce infection in the albino rat comparable in intensity to those with *T. lewisi*. On the other hand, the cotton rat trypanosome appears to be immunologically

similar to *T. lewisi*. Young albino rats on recovery from mild infections with the cotton rat trypanosome are immune to *T. lewisi* and the prior inoculation of cotton rat with *T. lewisi* appears to enhance its resistance to a subsequent infection with the cotton rat trypanosome. This immunological data suggests a close taxonomic relationship between *T. lewisi* and the cotton rat trypanosome, the latter perhaps representing a physiologically distinct strain of the *lewisi* type of trypanosome. Because, however, there are differences in morphology as well as in infectivity from the type species, it seems reasonable to ascribe to the cotton rat trypanosome a new specific name. The name *Trypanosoma sigmodoni* is, therefore, suggested as an appropriate name for the trypanosome of the cotton rat.

Trypanosoma sigmodoni n. sp.

Specific diagnosis: Protomonadida, Eumonadea, Trypanosomidae. Genus *Trypanosoma*. Characteristics of the genus. This species characteristically presents its nucleus only slightly if at all forward of the cell center, and its somewhat prominent kinetoplast near the pointed posterior end of the cell. There is a well-developed free flagellum, and the usual undulating membrane. Division stages have not as yet been seen in either the blood or the fixed tissues. The trypanosome is small, measurements on 25 parasites being: $14.8\text{ }\mu\text{--}20.0\text{ }\mu \times 1.6\text{ }\mu\text{--}2.5\text{ }\mu$; mean $16.9\text{ }\mu \times 2.0\text{ }\mu$ (all measurements exclusive of free flagellum and of undulating membrane). The species produces experimental infections of very low intensity in young albino rats, but does not infect guinea pigs, rabbits, or albino mice.

Host: *Sigmodon hispidus littoralis* (Mammalia, Rodentia). Florida cotton rat.

Habitat: Blood.

Locality: Englewood, Florida.

SUSCEPTIBILITY OF THE FLORIDA COTTON RAT TO TRYPANOSOMES OF
OTHER HOSTS

Cotton rats were tested for susceptibility to *T. equiperdum*, *T. lewisi*, and *T. duttoni*. The animals were found to be about as susceptible as albino rats to *T. equiperdum*. For example, one cotton rat was dead of the infection, its blood swarming with *T. equiperdum*, 5 days after the parasite was injected (Table 4). *T. lewisi* from the rat and *T. duttoni*

TABLE 4.—Course of infection with *T. equiperdum* in the cotton rat, and in the albino rat

Host	Parasite counts, on designated days after infection				
	1	2	3	4	5
Cotton rat	+	+	2+	4+	Dead
Albino rat	+	++	3+	4+	Dead

Key to parasite counts:

+ = less than 1 parasite per microscope field ($\times 430$);

2+ = from 1 to 5 parasites per field;

3+ = from 5 to 25 parasites per field;

4+ = over 50 parasites per field.

from mice failed to infect the cotton rat, no parasites being found at any time after inoculation, although the appropriate control animals given these parasites promptly became infected.

DISCUSSION

The cotton rat trypanosome appears to be another species of the *lewisi* group of trypanosomes. It differs from others of the *lewisi* group in several rather significant respects, but resembles the type species immunologically. Perhaps the most significant point of similarity is that recovery from a mild infection with the cotton rat trypanosome will completely protect a young albino rat against *T. lewisi*. Only nursing albino rats have proved susceptible to infection with the cotton rat trypanosome; it seems possible that nursing cotton rats (which were not available for the research) might likewise have contracted demonstrable infections with *T. lewisi*.

There is some interest among investigators at present as to whether the cotton rat is immunologically more like the albino rat or the guinea pig. It has been found to be more like the guinea pig in its susceptibility to diphtheria toxin (2), and tuberculosis (3), but like the rat in its refractoriness to anaphylactic shock (4). The high susceptibility and early death of the cotton rat from the pathogenic trypanosome *T. equiperdum* ranges this host with the rat rather than with the guinea pig with respect to its capacity for resistance against this trypanosome infection.

SUMMARY

A trypanosome found naturally in the peripheral blood of the Florida cotton rat, *Sigmodon hispidus littoralis*, is described. The name *Trypanosoma sigmodoni* is suggested for the parasite. The organism has been found in the blood of 18 (20%) of 90 Florida cotton rats examined. Infections can be readily induced artificially in normal cotton rats by inoculating them with the blood from an infected animal. Following a prepatent period of 6 to 10 days after the injection of infective material, the parasites are found in the peripheral blood for about one month. Infections generally do not rise above an intensity of 25,000 organisms per cubic millimeter of blood, and none of the animals studied has died of its infection. Nursing albino rats are susceptible to very mild infections with the cotton rat trypanosome.

On recovery from infection with this trypanosome, the cotton rat is immune to reinfection, and its serum inoculated to a normal cotton rat protects the normal animal from infection. The trypanosome appears to be somewhat closely related immunologically with *T. lewisi*, since young albino rats which have recovered from an experimentally induced infection with the cotton rat trypanosome are protected against *T. lewisi*. Likewise, a cotton rat previously inoculated with *T. lewisi* acquires a partial immunity against the cotton rat trypanosome.

The cotton rat is highly susceptible to *T. equiperdum*, succumbing to infection with this species in about 5 days. The cotton rat resists infection with *T. lewisi* and *T. duttoni*.

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THE PENETRATION OF RADIOACTIVE PHOSPHORUS INTO ENCYSTED *TRICHINELLA* LARVAE

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It is well-known that *Trichinella spiralis* larvae can live for an extraordinarily long time encysted in the muscles of a host. There are many reports in the literature describing larvae which have been found alive in human muscle, for from 5 to 30 years after the disease was thought to have been contracted (1, 2, 3, etc). In the authors' experience, numerous living larvae were found in a muscle removed at biopsy from a man who had had a very severe attack of trichiniasis seven and one-half years previously. This man had had the cause of his illness explained to him and had been so afraid of contracting the disease a second time that he claimed that he had not eaten meat of any kind since. It seems probable, therefore, that the larvae found in this case must have been present since the original illness.

The generally accepted view, as expressed many years ago by Staubli (4), is that, after encystment, *Trichinella* larvae enter a latent stage in which they are walled-off from the tissues of the host. It is true that the larvae do not grow or develop while encysted, but it is well-known that they are capable of movement within their cysts. Stannard, McCoy and Latchford (5) showed that larvae digested free from their cysts exhibit both an aerobic and an anaerobic metabolism when kept in Tyrode's solution at 37° C. It is not known whether encysted *Trichinella* larvae live on food materials stored within their own bodies, or whether they absorb nourishment through the cyst wall from the host. In view of the long periods of time during which they remain viable in the encysted stage, the latter seems the more probable. The present preliminary experiments are designed as a test of the permeability of the cyst wall.

Within the past few years, the production of radioactive isotopes of such elements as sodium, potassium, phosphorus, iron, etc, has afforded the means for tracing the distribution of these substances in the bodies of experimental animals. This method of study, of course, is equally applicable to parasites which may be present in these animals. In the present experiments, tests were made of the ability of encysted *Trichinella*

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larvae to absorb radioactive phosphate ions. Phosphorus was selected as the test substance because a source of the radioactive element was readily available, and because the half-life of radioactive phosphorus is relatively long (14.5 days), thus convenient for experiments lasting for several days or more.

MATERIALS AND METHODS

The tests were made upon laboratory bred albino rats of Wistar stock which had been heavily infected with *Trichinella spiralis* from 8 to 10 months previously. After this period of time, the larvae were enclosed in thick-walled cysts of hyalinized connective tissue characteristic of long-standing muscle infection. (Fig. 1.) No calcification, however,

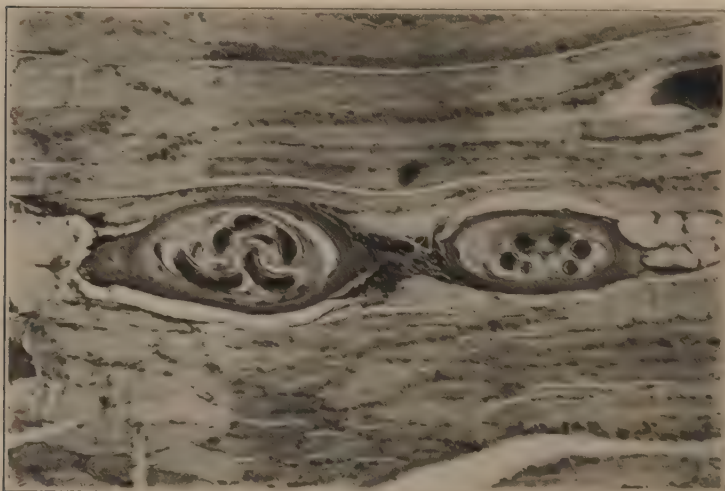


FIG. 1. Encysted *Trichinella spiralis* larvae in the muscle tissue of a rat killed 8 months after infection (magnification $\times 100$).

was observed. It was considered that cysts of this age and degree of development would offer a fair test of the permeability of *Trichinella* cysts in general.

The rats were fed by stomach tube 1-cc amounts of a solution containing radioactive phosphorus in the form of basic sodium phosphate. They were killed thereafter at intervals varying from 2 hours to 6 days. After removing 3-gm samples of muscle for analysis, the carcasses were digested for 3 hours at 37°C with a solution of 0.7 per cent pepsin and 0.5 per cent hydrochloric acid. The larvae freed from their cysts were collected by sedimentation, washed free from debris, desiccated and weighed.

The amount of radioactive phosphorus present in the larvae was de-

terminated by the use of a Geiger-Müller counting apparatus according to the method described by Bale, Haven and LeFevre (6). The amount of radioactive phosphorus is calculated in terms of the percentage of the original dose fed to the rats which was determined per gram of phosphorus present in the larvae. This figure is called the "specific radioactivity" of the larvae and enables a comparison to be made with the amount of radioactive phosphorus present in the muscles. To calculate this figure it was, of course, necessary to determine the amount of phosphorus in each sample of larvae and of muscle.²

In the procedure followed for recovering the larvae from the infected muscle, it was possible that the larvae might take up radioactive phosphorus after being digested free from their cysts. In order to test this possibility, the carcasses of uninfected rats which had been fed radioactive phosphorus were ground up and digested according to the same procedure used for the experimental animals. At the time digestion was begun, however, normal larvae, recovered from rats not fed radioactive phosphorus, were added to the digestion fluid. When tested subsequently, it was found that these larvae did not contain a measurable amount of radioactive phosphorus. Two tests of this nature were performed in each of the three experiments. It seems reasonable to conclude, therefore, that the radioactive phosphorus detected in the larvae must have been absorbed while they were still within their cysts.

EXPERIMENTAL

Experiment 1. Eight female rats infected with *Trichinella* 10 months previously were used. They were killed in pairs at intervals of 1, 2, 4 and 6 days after feeding radioactive phosphorus. The results are tabulated in Table 1. A significant amount of radioactive phosphorus was detected in the larvae from rats killed after 24 hours, and the greatest relative amount was present in the larvae from those killed after 4 days.

Experiment 2. Six male rats infected for 8 months were killed in pairs at intervals of 12, 24 and 48 hours after feeding radioactive phosphorus. The results, which are also tabulated in Table 1, support those found in the first experiment. The larvae recovered from one of the rats (No. 9) killed after 12 hours showed an abnormally large amount of radioactive phosphorus in comparison with that found in the larvae recovered from the other animals. This figure, therefore, is omitted from the averages. The larvae from the other rat killed after 12 hours contained almost as much radioactive phosphorus as the average amount found in the larvae from the two rats killed after 24 hours.

Experiment 3. Ten male rats infected 8 months previously were killed in pairs at intervals of 2, 4, 8, 12 and 24 hours after feeding radio-

² The writers are indebted to Dr. Richard S. Manly and Miss Sylvia Levy, Department of Biochemistry, for performing these analyses.

TABLE 1.—Amount of radioactive phosphorus present in *Trichinella* larvae and the muscles from rats killed at intervals after feeding radioactive phosphorus

Rat No.	Interval between feeding P and death	Dry weight of larvae recovered (in mgm)	Per cent of phosphorus in larvae	Specific radioactivity of larvae*	Wet weight of muscle sample (in gm.)	Per cent of phosphorus in muscle	Specific radioactivity of muscle*
<i>Experiment 1.—Female rats infected for 10 months</i>							
1	24 hr.	115	0.67	11.7	3.156	0.18	5.6
2	24 "	80	0.69	12.3	3.107	0.18	5.5
3	48 "	68	0.69	26.4	2.682	0.18	6.2
4	48 "	205	0.71	13.5	2.935	0.18	5.7
5	4 days	130	0.40	24.9	2.840	0.20	7.6
6	4 "	55	0.84	24.6	2.865	0.21	6.9
7	6 "	100	0.64	18.5	3.050	0.18	4.8
8	6 "	95	0.70	20.9	3.122	0.18	5.8
<i>Experiment 2.—Male rats infected for 8 months</i>							
9	12 hr.	102	0.76	70.6†	2.567	0.21	19.4
10	12 "	94	0.67	14.3	2.632	0.23	28.1
11	24 "	90	0.57	19.3	2.582	0.22	21.3
12	24 "	115	0.78	11.5	2.705	0.23	13.4
13	48 "	87	0.54	20.3	2.413	0.22	16.7
14	48 "	76	0.67	11.5	3.264	0.22	16.7
<i>Experiment 3.—Male rats infected for 8 months</i>							
15	2 hr.	153	0.74	0.86	2.232	0.22	7.22
16	2 "	215	0.66	1.08	3.290	0.21	6.04
17	4 "	174	0.80	2.71	3.038	0.19	7.30
18	4 "	184	0.70	3.06	4.159	0.18	7.36
19	8 "	169	0.74	2.68	3.816	0.22	9.44
20	8 "	195	0.71	3.92	3.094	0.19	6.59
21	12 "	165	0.71	6.44	3.848	0.23	9.38
22	12 "	251	0.76	5.45	2.735	0.23	8.83
23	24 "	126	0.45	4.99	3.045	0.20	8.70
24	24 "	239	0.34	19.20	2.423	0.21	11.60

* "Specific radioactivity" is the amount of radioactive phosphorus present per gram of phosphorus expressed as the percentage of the original dose fed.

† This figure not included in averages.

‡ Sample lost.

active phosphorus. The results are also shown in Table 1. The amount of radioactive phosphorus found in the larvae recovered from the rats killed after 2 hours was about the minimum that could be detected by the method employed. Increasing amounts were found in the larvae from the rats killed after from 4 to 24 hours.

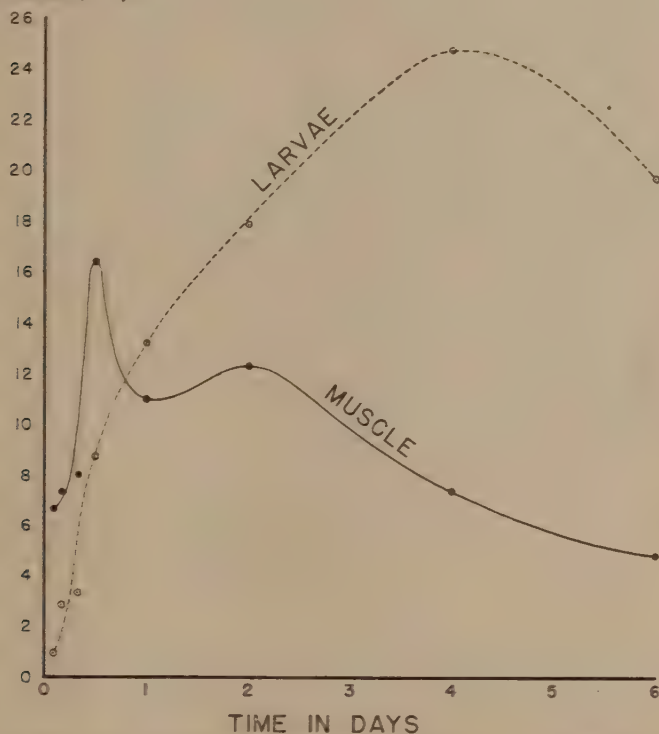
DISCUSSION

The "specific radioactivity" of the larvae recovered from pairs of rats killed at the same time showed considerable variation in all three experiments. Experimental error in desiccating and weighing the larvae, in analyzing their phosphorus content and in making the counts probably accounts for some of this variation. In addition, there appears to be an inherent variability in the different lots of rats that were used. For instance, the "specific radioactivity" of the muscles of the rats in Exper. 1 was much less than that measured in the other two experiments. The fact that these rats were of a different sex and were two months older than the rats in the other experiments may account for this difference. It should be mentioned, also, that the three experiments were performed at different seasons of the year.

The figures for the "specific radioactivity" of the larvae and of the muscles from rats killed at the same intervals after feeding radioactive phosphorus were averaged for the three experiments and plotted (Fig. 2). The individual points represent the average results in from 2 to 6

SPECIFIC RADIOACTIVITY

(% OF FED DOSE
PER GRAM OF P)



(POINTS ARE AVERAGES OF RESULTS IN FROM 2 TO 6 ANIMALS)

FIG. 2. The relative amount of radioactive phosphorus found in *Trichinella* larvae and muscles from rats killed at intervals of from 2 hours to 6 days after feeding. The individual points are the averages of the results in from 2 to 6 animals. The curves are drawn free-hand and fitted by inspection.

animals. The curves were drawn free-hand and fitted by inspection. Caution must be used in interpreting these curves because of the variability of the material on which they are based. It appears, however, that the rate of absorption and retention of radioactive phosphorus followed a

course different from that for the muscles. The radioactive phosphorus was taken up more rapidly by the muscles and was also lost more rapidly.

The data available in the present experiments are not sufficient to indicate whether the absorption of radioactive phosphorus by the larvae represents an actual metabolic exchange or merely a diffusion of phosphate ions into the larvae. The rapidity of absorption by the encysted larvae, however, might indicate that they are undergoing active metabolism. In any event, it is established that the fully developed cyst wall surrounding *Trichinella* larvae in muscle tissue is permeable to phosphate ions.

SUMMARY

In three experiments, 28 rats, heavily infected from 8 to 10 months previously with *Trichinella spiralis*, were fed radioactive phosphorus (as basic sodium phosphate) and killed at intervals beginning 2 hours later. Radioactive phosphorus was detected in the larvae as early as 2 hours after feeding and the amount increased rapidly during the first 24 hours. The maximal content was reached on the fourth day, after which there was a gradual decline. The absorption and loss of radioactive phosphorus by the larvae follow a course different from that for the hosts' muscles. The experiments demonstrate that a ready exchange of phosphate ions may take place through the cyst wall surrounding *Trichinella* larvae in the muscles of rats, and imply that the larvae may be undergoing active metabolism during the encysted stage.

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AMPHIBIAN NEMATODES FROM THE GASPÉ PENINSULA AND VICINITY

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Through the courtesy of Dr. J. S. Rankin, Jr., of Amherst College, a number of nematodes from amphibian hosts, collected during a trip to the Gaspé Peninsula in June, 1938, were sent to the writer for identification. A number of new locality records for several known species of nematodes are hereby added to the literature, as well as the description of a new species of the genus *Spironoura* Leidy, 1856.

Rana pipiens (5 specimens from Matapedia, Gaspé Peninsula) is a host for *Cosmoceroides dubus* (Holl, 1928) Wilkie, 1930.

Rana clamitans (8 specimens from near Matapedia, Gaspé Peninsula) is a host for *Foleyella ranae* Walton, 1929, and for an *Oxyssomatium* species. Only immature females of the latter were present, so no species designation is attempted.

Rana clamitans (6 specimens from Madelene, Gaspé Peninsula) is a host for *Cosmoceroides dubus* (Holl, 1928) Wilkie, 1930.

Rana clamitans (2 specimens from Chatham, New Brunswick) is a host for *Foleyella americana* Walton, 1929.

Rana clamitans (2 specimens from Machias, Maine) is a host for *Foleyella ranae* Walton, 1929, *Oswaldocruzia leidy* Travassos, 1917, and *Spironoura rankini* n. sp.

Rana pulchris (1 specimen from Machias, Maine) is a host for *Oswaldocruzia leidy* Travassos, 1917.

Bufo americanus (1 specimen from Machias, Maine) is a host for *Oswaldocruzia pipiens* Walton, 1929.

Spironoura rankini n. sp.

(Fig. 1)

Description: Based on male material only. With the characters of the genus: head with three well-developed lips bearing two outer and two inner papillae; oral cavity supported by a cuticular ring; pharynx muscular; esophagus with terminal bulb and a pre-bulbar swelling; lateral fields wide; lateral and caudal alae absent; pre-cloacal musculature well developed; spicules sickle-shaped and alate; accessory piece present. Length, 20.15 mm; greatest width, 0.375 mm; length of pharynx, 0.112 mm; length of esophagus, 1.45 mm; pre-bulbar swelling, 0.225 mm in length and 0.075 mm in width; bulb spherical, 0.185 mm in diameter; head-nerve ring distance, 0.06 mm; head-excretory pore distance, 1.5 mm; cloaca-tail distance, 0.375 mm; spicule length, 1.05 mm, laterally compressed and with narrow unilateral alae; accessory piece length, 0.112 mm; 3 pairs of pre-cloacal papillae, 4 pairs of para-cloacal papillae, and 3 pairs of post-cloacal papillae, in addition to the unpaired median papillus on the anterior margin of the cloaca.

Host: *Rana clamitans* collected June 22, 1938, at Machias, Maine).

Habitat: Intestine.

Type specimens: Co-types deposited in U. S. Nat. Mus. Helm. Coll.

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* Contribution from the Biological Laboratories of Knox College, No. 67.

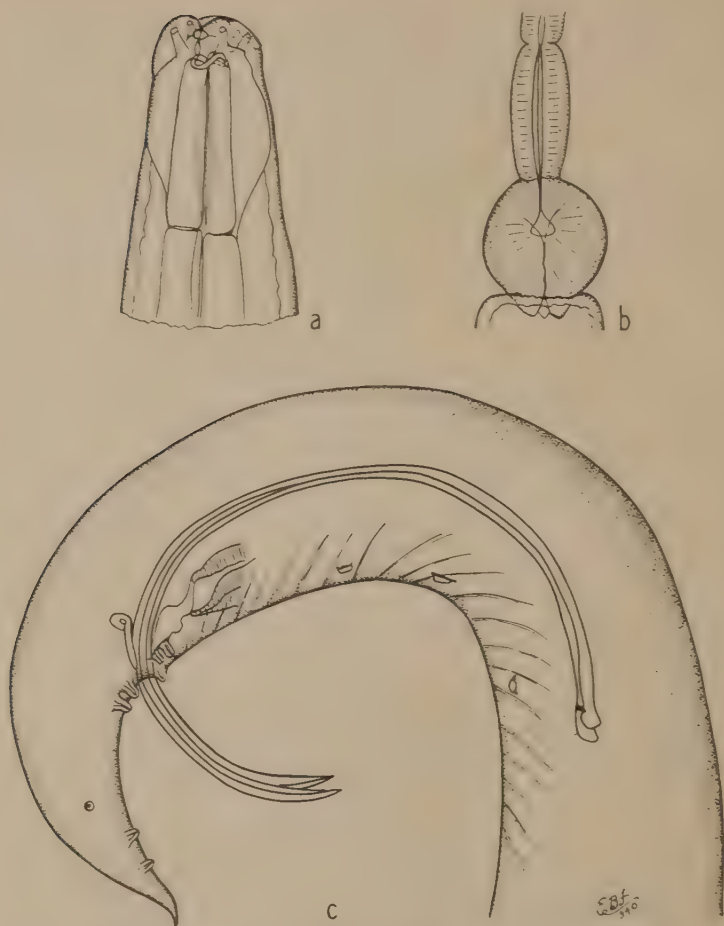


FIG. 1. *Spironoura rankini*. a. Head end of male.
b. Esophageal bulb from male.
c. Lateral view of tail of male.

The present species, while much larger than the other species reported from North American amphibia, most nearly resembles *Spironoura elongata* (Baird, 1858) Walton, 1932, from *Ambystoma tigrinum*, and *S. pretiosa* Ingles, 1936, from *Rana pretiosa*, but differs in the relative proportions of the structures at the anterior end, in the sizes of the spicules and accessory piece, and in the number and arrangement of the caudal papillae. *S. rankini* is more than twice the length of any of the species except *S. elongata* and *S. pretiosa*, and is a third longer than adult males of these two species. The specific name is in honor of the collector of this material.

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TWO NEW MESOZOA FROM CALIFORNIA, *DICYEMENNEA CALIFORNICA* AND *DICYEMENNEA BREVICEPHALA* (DICYEMIDAE)*

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Our knowledge of the DICYEMIDAE (MESOZOA) is almost entirely embodied in several excellent works on the European species. Those of the Pacific Ocean have been neglected and are unknown except for the work of Dr. Wm. Morton Wheeler (1897), who named three species found at San Diego, California, but did not describe or figure them, and that of Nouvel and Nakao (1938) who described a few species of *Dicyema* from Japan. During the summers of 1937 and 1939 and in December of 1938 I examined the kidneys of several octopi from the littoral zone in the Laguna Beach area and of one from the San Francisco Bay area. All the octopi examined except those a centimeter or less in length, were richly infected. The two most abundant species are herein described.

Dicyemennea californica, n. sp.

(Figs. 1, 2, 3, 6, 8)

Specific diagnosis: Body attenuate, vermiform individuals mostly 1.7 to 3.0 mm long. Calotte enneamerous (4 propolar cells, 5 metapolar cells), conical, forming about a third to over half the cephalic swelling in the adult. Parapolar cells shield shaped forming from about half to two thirds of the cephalic swelling. Total number of peripheral cells 36 or less (primary rhombogenes with 31). Uropolar cells usually swollen and vacuolated distally. Cilia nearly uniform, slightly denser on the calotte than over the trunk. Infusoriform individuals 38 to 45 micra in length, rounded posteriorly.

Nematogenes usually 25 to 50 micra wide, broadest at the level of the parapolar cells. Peripheral cells 36 in number. Calotte conical, orthotropical, made up of four propolar cells (two dorsal and two ventral), and five metapolar cells (one dorsal, two dorso-lateral, two subventral). Parapolar cells shield shaped, lateral, their broad anterior ends encircling the axial cell just behind the calotte.

Transitional stages from nematogene to rhombogene with greatly increased number of agametes in the axial cell, which is broader than in the nematogene, and often contains simultaneously more or less advanced vermiform larvae and one or more young infusorigenes. Vermiform larvae in such cases mostly with the reduced number of cells (31).

Rhombogenes noticeably broader and more robust in appearance than the nematogenes. Primary rhombogenes with 31 instead of 36 peripheral cells, the nuclei of the trunk cells often enlarged and sometimes constricted in two giving rise to accessory nuclei in some of the peripheral cells. Accessory nuclei also frequently occur in the axial cell in this stage.

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*The writer wishes to express his appreciation to Dr. W. A. Hilton of Pomona College for the use of the facilities of the Pomona College Marine Laboratory at Laguna Beach, to Dr. S. S. Berry of Redlands University for determining the host species of *D. brevicephala*, and to Dr. S. F. Light and Dr. Harold Kirby, Jr. of the University of California for aid and advice in the presentation of this material.

Infusoriform individuals 38 to 45 micra in length when fully developed, rounded posteriorly.

Host: *Octopus bimaculatus* Verrill.

Distribution: Southern California coast, taken in the littoral zone at Laguna Beach, Emerald Bay and Balboa Bay, Orange Co., Calif.

Type specimens: U. S. Nat. Mus. No. 22682, paratype No. 22683, other paratypes in Univ. Calif. Coll. and of author.

Dicyemenea brevicephala, n. sp.

(Figs. 4, 5, 7, 9, 10)

Specific diagnosis: (There has been less material of this species available to me than of *D. californica*, all of it being from one octopus taken at Marine View Rocks, San Mateo Co., Calif., early in June 1939. Practically all the dicyemids were mature rhombogenes.)

Medium sized to small dicyemids, mostly 1 to 2 mm long. Total number of peripheral cells 27, this probably the number for primary rhombogenes. Calotte enneamerous, short, wide, appearing in many cases as a flattened disc at the truncate ends of the elongated parapolar cells. Metapolar cells usually from two to five times as large as the propolar cells, short, frequently wider than long. The cilia of the calotte shorter and more closely set than those of the trunk cells. Parapolar cells usually very elongate, forming most of the cephalic swelling, generally 100 to 150 micra in length. Uropolar cells not often swollen and vacuolated as in *D. californica*, quite similar in appearance to the other trunk cells. Older rhombogenes frequently much swollen anteriorly, the cells of the calotte becoming enlarged and displaced obscuring their typical arrangement, anterior end of axial cell also swollen in these cases. Infusoriform individuals 28 to 35 micra in length when fully developed, rounded posteriorly.

Host: *Octopus apollyon* (Berry).

Distribution: Known only from Marine View Rocks, San Mateo Co., Calif.

Type specimens: U. S. Nat. Mus. No. 22680, paratypes No. 22681, other paratypes in the Univ. Calif. Coll. and of author.

The two species here described differ in several respects from other known *Dicyemenea*. *D. lameerei* Nouvel, *D. eledones* Wag. and *D. gracile* Wag. each possess only 23 peripheral cells. *D. eledones* and *D. gracile* are both larger species, ranging from 4 to 7 mm in length, and their parapolar cells do not contribute much to the cephalic enlargement. *D. lameerei* tends to be smaller and more massive, ranging from 1 to 1.5 mm in length, and is further distinguished by the constant presence of fine crescentic pointed crystalloid needles in the cytoplasm of the peripheral cells and by the fact that the infusoriform is pointed posteriorly and has only one nucleus and one germ cell in each of the cells of the urn. The host of *D. lameerei* is *Octopus vulgaris* Lam., those of *D. eledones*, *Eledone moschata* Leach and *E. aldroandi* D. Ch., and that of *D. gracile*, *Sepia officinalis* L.

Dicyemenea mulleri Clap. was described by Claperède from specimens taken from *Eledone cirrosa* Lk. along the coast of Norway. As neither the total number of peripheral cells nor the number of cells in the calotte was determined its status remains in doubt. *D. whitmanii* Wheeler is a nomen nudum, no description or figures of it having been published.

Transitional stages between the nematogene and rhombogene phases of *D. californica* were found. These show a great multiplication of the agametes. Several individuals contained advanced embryos of the vermiform stages and one or more young infusorigenes and in some cases even very young infusoriform embryos in the axial cell simultaneously. The young infusorigenes already have the small scattered chromatic bodies always found associated with the infusorigenes of dicyemids. These have been interpreted as sperm by Wheeler (1897), Hartmann (1906), Lameere (1918) and Nouvel (1933), and as irregularly scattered chromosomes resulting from abnormal divisions of the cells of degenerating pycnotic infusorigenes near the end of the rhombogene phase by Gersch (1938). In my material they are found associated both with the very young infusorigenes in transforming individuals before any infusoriforms have been formed and with the older infusorigenes. The cells of the younger infusorigenes do not show evidence of pycnosis, and the chromatic bodies are larger than the individual chromosomes seen in the cells and are quite constant in shape. I have as yet, however, not been able to determine definitely their exact mode of origin and am not fully satisfied that they are sperm.

Nouvel (1938) called attention to a very curious point in the embryology of the vermiform individuals in *Dicyemennea lameerei* and *D. eledones*, in which the first germ cell of the developing larva upon division gives rise to two cells, one of which becomes the mother cell of all the future germ cells or axoblasts and the other of which takes a position at the anterior end of the axial cell, then invades the dorsal metapolar cell of the embryo where it remains, finally degenerating and disappearing completely after the larva escapes from its parent.

This phenomenon is very clearly shown in my material in both species and in both the nematogene larvae and those destined to become primary rhombogenes. It is probably general in *Dicyemennea* but has not been noted in any species of the related genus *Dicyema* the embryology of which has been carefully investigated by several authors.

SUMMARY

Two species of *Dicyemennea* from the kidneys of octopi of the littoral zone of the California coast are described. They differ from other known *Dicyemennea* in cell number, size, composition of the cephalic swelling and other details.

Some evidence against Gersch's theory of the origin of the sperm-like chromatic bodies associated with the infusorigene is presented.

The phenomenon described by Nouvel wherein one of the cells resulting from the first division of the primary germ cell in the vermiform embryo invades the dorsal metapolar cell and degenerates there has been confirmed. It is probably general in *Dicyemennea*.

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EXPLANATION OF PLATES

All figures drawn with the aid of camera lucida

PLATE I

FIG. 1. Calotte of *Dicyemenea californica*, late nematogene. (Bouin—Jansens haematoxylin.)

FIG. 2. Primary rhombogene larva in axial cell of an individual transitional between the nematogene and rhombogene phases, showing the reduced number of peripheral cells (31) and the degenerating germ cell in the dorsal metapolar cell just anterior to the end of the axial cell. (Bouin—Ehrlich's haematoxylin.)

FIG. 3. Nematogene larva in the axial cell of a primary nematogene, showing the 36 peripheral cells and the degenerating anterior germ cell. (Bouin—Ehrlich's haematoxylin.)

FIG. 4. Calotte of *Dicyemenea brevicephala*, mature rhombogene. (Bouin—borax carmine, indulin.)

FIG. 5. Vermiform larva of *D. brevicephala* (probably a primary rhombogene) showing 27 peripheral cells and the degenerating anterior germ cell. (Bouin—borax carmine, indulin.)

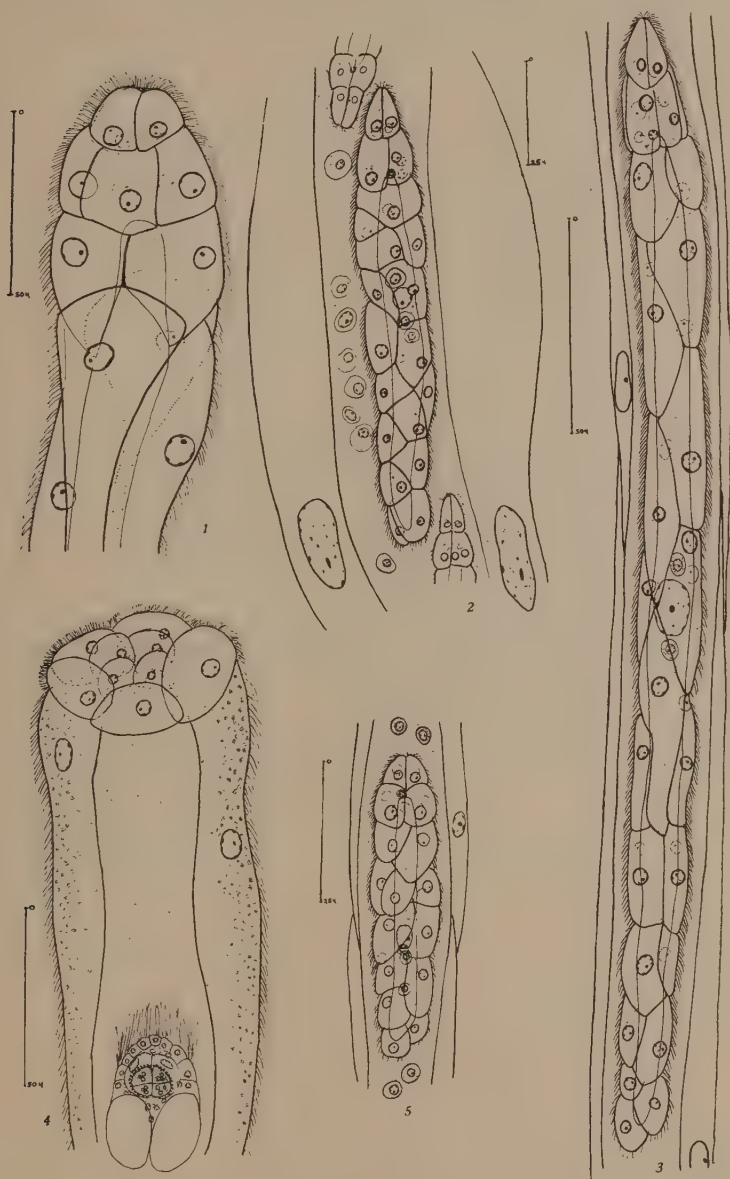


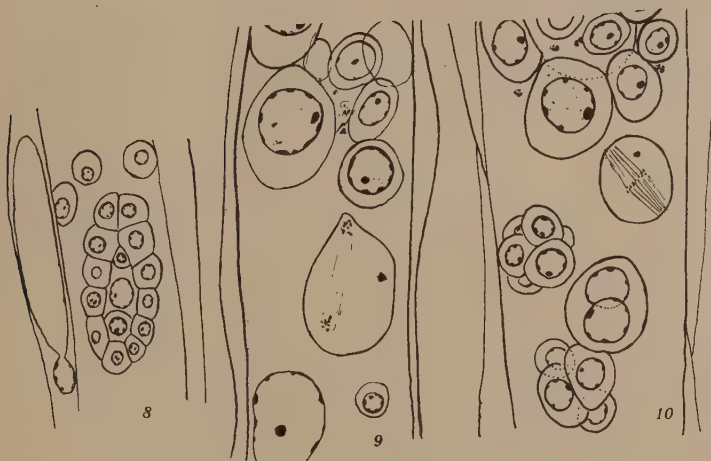
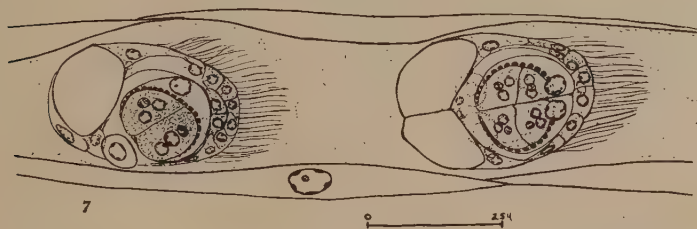
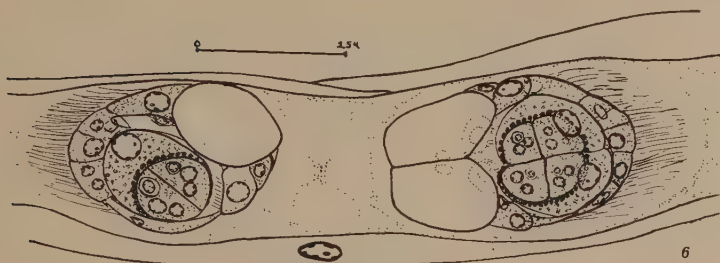
PLATE II

FIG. 6. Infusoriform of *D. californica* parasagittal and frontal optical sections. (Bouin—Ehrlich's haematoxylin.)

FIG. 7. Infusoriform of *D. brevicephala*, same views. I think there should be cilia in the cavity of the urn here too but was unable to see them clearly in this case. (Bouin—Jansens haematoxylin.)

FIG. 8. Very young vermiform of *D. californica*, about 22 cell stage, in optical section, showing the axial cell and the two cells resulting from the first division of the primary germ cell, one, posterior and larger, the mother cell of all the future axoblasts; the other, anterior and smaller, destined to invade the dorsal metapolar cell and degenerate there. (Bouin—Ehrlich's haematoxylin.)

FIGS. 9 and 10. Portions of infusorigenes and division of the "egg" cells in *D. brevicephala*, showing conditions cited by several authors as evidence of sperm penetration and polar body formation. In Fig. 10 there is also shown an "egg" cell with two nuclei which are regarded by most authors as the male and female pronuclei before the first cleavage but by Gersch (1938) as merely two nuclei resulting from failure of the cytoplasm to divide following nuclear division. These stages are relatively infrequent in my preparations but where they occur they are almost always in the same order passing from the infusorigene outwards. (Bouin—Jansens haematoxylin.)





THE INCIDENCE OF INTESTINAL PARASITES IN A SELECTED GROUP AT A MENTAL HOSPITAL*

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This report is of a survey conducted to determine the incidence of parasites in a selected group of patients. The cases examined were all white women of varying ages but about the same mental level, untidy in their habits, and confined in a separate building with semiprivate recreation grounds.

Strict hygienic measures have been employed during the residence of the patients. The building is kept in good repair, paying particular attention to the sealing of all small openings where filth and contamination might collect. The floors are of glazed tile. The recreation grounds consist of two separate yards: these are limed, worked over and resodded with new grass and each yard is unoccupied for alternate six months intervals. Even with such measures, the problem of parasitism is still acute.

MATERIALS AND METHODS

Examinations were made of freshly obtained fecal specimens which were collected in one-half pint cardboard containers. Two specimens were examined from all patients, but some, especially those found to be infected with *Balantidium coli*, were repeatedly examined. Simple saline and saline-iodine smear preparations were used. No technique for concentration of parasites was employed. It is recognized that concentration techniques will reveal infections not found by the simple smear method and therefore the incidence should be considered as a minimum for the group. Undoubtedly many low grade infections were not detected.

Following the stool examinations, the patients were given anthelmintics. It should be borne in mind that these patients have been treated in the past at regular intervals. At the time of each periodic medication numerous parasites were eliminated.

OBSERVATIONS

One hundred forty-two patients were examined and parasites were found in 128 or 90 per cent. One hundred seventeen (82 per cent) harbored helminths and 92 (65 per cent) were infected with protozoa.

The number of species of parasites harbored by the patients were as follows: 14 patients had no species of parasites; 11 had one; 22 had 2;

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35 had 3; 29 had 4; 15 had 5; 10 had 6; 5 had 7; and one had 8 species. This gives a total of 454 specific infections in the 142 patients or an average of 3.2 species per person.

Table 1 shows the distribution of parasites by species and grouped according to the length of residence of the patients in the hospital.

TABLE 1.—Distribution of parasites according to the length of residence of the patients in the hospital

Years in Hospital	Less than one	1-5	6-10	11-15	16-20	21-25	26-30	31 +	Total
No. examined	6	44	24	32	17	7	8	4	142
Infected	%	%	%	%	%	%	%	%	No. %
<i>Strongyloides stercoralis</i>	17	39	41	41	50	29	38	50	56 39
Hookworm	17	66	71	59	77	43	100	100	94 66
<i>Trichuris trichiura</i>	50	73	75	78	94	57	88	100	111 78
<i>Ascaris lumbricoides</i>	17	7	13	9	0	14	13	50	14 10
<i>Endamoeba histolytica</i>	0	3	4	7	0	14	0	0	5 4
<i>Endamoeba coli</i>	33	45	57	50	50	43	25	100	68 48
<i>Endolimax nana</i>	17	20	35	28	19	14	38	0	34 24
<i>Dientamoeba fragilis</i>	0	0	4	0	0	0	13	0	2 1
<i>Iodamoeba bacilliformis</i>	0	11	17	7	0	14	13	0	13 9
<i>Trichomonas hominis</i>	17	14	26	28	19	14	0	50	28 20
<i>Giardia lamblia</i>	17	5	4	7	0	14	25	0	9 6
<i>Chilomastix mesnili</i>	16	8	13	7	6	14	0	0	11 8
<i>Retortamonas intestinalis</i>	0	0	0	3	0	0	13	0	2 1
<i>Balantidium coli</i>	0	3	9	7	6	0	13	0	7 5
Number of patients infected with helminths	67	82	90	72	94	57	100	100	117 82
Number of patients infected with protozoa	33	64	83	63	59	57	50	100	92 65

Trichuris trichiura showed the highest rate of infection, an incidence of 78 per cent. This incidence is similar to that of Caldwell, Caldwell and Davis (1930) who found between 80 and 90 per cent infection with trichurids in the "untidy" patients at the Alabama State Hospitals. As will be seen from the table the trichurid infections apparently increased with the duration of hospital residence. This conclusion is further borne out by comparing the incidence in this series with the results obtained by Leathers and Keller (1937) of 0.04 per cent for the state as a whole.

Hookworm infection rate for the state of South Carolina during 1934-1935 was found by Leathers, Keller and Wyman (1936) to be 24.8 per cent. In our series, 66 per cent were infected. This rate and the gradual rise of the incidence during hospital residence indicates that the infection increased during hospitalization.

The *Ascaris lumbricoides* infection was 10 per cent, which compares favorably with the findings of Leathers and Keller (loc. cit.) who found

an incidence of 4 per cent in the state with the rate varying from 0.02 to 11.4 per cent. Apparently, little increase in this infection occurred with hospital residence. In the Alabama mental institutions, Caldwell, Caldwell and Davis (loc. cit.) found that ascariasis did not increase as rapidly as trichuriasis.

The *Strongyloides stercoralis* infections were diagnosed by finding typical rhabditiform larvae in the freshly passed stools. In addition, some of the stools were cultured in bone charcoal; characteristic filariform larvae and adults were recovered from these cultures, both by simple smears and by the use of the Baermann apparatus. Dr. George L. Graham, of the Rockefeller Institute for Medical Research, confirmed the diagnosis of the rhabditiform larvae as *S. stercoralis* and for this courtesy we wish to express our appreciation.

S. stercoralis infections were found in 56 of the 142 patients examined, an incidence of 39 per cent. The authors have no information on the incidence of strongyloidiasis in South Carolina but, compared to reports from other localities, the 39 per cent infection found here appears to be high.

No infections of *Enterobius vermicularis* were found. However, as has been shown by several investigators, stool examinations do not reveal the true rate of incidence of this parasite.

Balantidium coli is of particular interest among the protozoan infections. This parasite was found in 7 cases of the 142 patients, an incidence of 5 per cent. These infections were reported in detail by Young (1939) and appear to be the first cases reported from South Carolina. As this rarely is reported under normal conditions, it seems that the hospital rate of 5 per cent indicates an increase during residence. All cases occurred in patients of very poor mental state, those who require constant supervision as to habits and general conduct.

DISCUSSION

A correlation of the parasitism to the type of psychoses did not reveal anything of significance, especially as the total numbers in the various groups were comparatively small.

The patients examined were housed in one building because of their behavior and general mental state rather than for any specific mental classification, so that the common denominator of the group is that of untidy habits. A description of some of the habits of the patients was given by Young (loc. cit.) in discussing the *Balantidium coli* infections as follows: "Because of their mental condition, all the infected patients tolerated untidiness, both personal and environmental. The clothing might become contaminated and often it was difficult to collect sufficient feces for an examination. This type of patient often puts rocks, leaves,

sticks, grass, dirt and other debris into his mouth. Thus the factors for the transfer of the infections from one person to another were present."

The incidence of parasites in this selected group emphasizes, as has been pointed out by other observers, that when conditions of soil and climate are favorable, any break in personal hygiene completes the cycle of the transmission and leads to a wide dissemination of certain intestinal parasites, especially helminths. It is recognized that such a break in hygiene is found in certain mental patients and as a result, contamination becomes a problem to be constantly guarded against. Many localities in the South have a soil and climate favorable for the development and dissemination of human parasites. In such areas, mental hospitals with their complement of untidy patients, are faced with a task of prime importance in preventive medicine.

SUMMARY

A group of patients, who because of mental deterioration were inclined to have untidy habits, were examined for intestinal parasites.

Of 142 examined, 128 (90 per cent) were harboring parasites; 117 (82 per cent) had helminths and 92 (65 per cent) had protozoa. Of these, the most important infections were: *S. stercoralis*, 39 per cent; hookworm, 66 per cent; *T. trichiura*, 78 per cent; *A. lumbricoides*, 10 per cent; and *B. coli*, 5 per cent.

Hookworm, strongyloides, trichuris, and balantidium appeared to increase with residence at the hospital. These findings emphasize again the difficult task in preventive medicine for mental hospitals located in a climate and soil area favorable to the dissemination of intestinal parasites.

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NATURAL TRANSMISSION OF IMMUNITY AGAINST *TRYPANOSOMA LEWISI* FROM VACCINATED MOTHER RATS TO THEIR YOUNG

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It has been shown by several studies previously reported from this laboratory that mother rats and mice, after recovery from trypanosome infections, transmit to their young specific protective substance. These substances are in all known respects similar to the antibody of the serum, and presumably are identical with it. They are passed to the young largely or entirely through the milk of the mother, and only in small amount, if at all, through the placenta. This last fact is supported by observations that (1) the young of normal animals, if let nurse an immune mother for a day or so, promptly become resistant; and (2) the young of the immune mother, if transferred promptly after birth to a normal mother before ingesting the milk of the immune mother, manifest little or no immunity. The immunity acquired by nursing an immune mother is of comparatively brief duration, being lost by the young wholly or largely within a few weeks after they are weaned (1-4).

The present paper offers the results of a study designed to test whether mother animals immunized not by recovery from infection but by vaccination with a formalized suspension of trypanosomes likewise transmit protective substances to the young in an amount sufficient to protect the young from the specific parasite.

MATERIALS

The strain of trypanosome and the preparation of the trypanosome vaccine.—*Trypanosoma lewisi* was employed in this experiment, the strain being supplied by Dr. L. Reiner of the Burroughs Wellcome and Company, Tuckahoe, New York. The vaccine was prepared in the manner described by Culbertson and Kessler (5): rats 15 days old when infected with *T. lewisi* were bled from the carotid when their infections were near the peak; the citrated blood was slowly centrifugated to separate out its trypanosomes; when freed of erythrocytes, the trypanosomes were suspended in 0.5 per cent formalized physiological sodium chloride solution, and their number determined by count with a hemocytometer. For injection, a suspension containing 4.3 million organisms per cubic centimeter of fluid was employed. The sterility of the vaccine was proved by inoculating young rats with it, with no infection ensuing.

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The strain of rats.—The rats were of the Sherman strain, propagated in the Department of Animal Care of this institution. They were fed the same diet, previously described (5), throughout the experiment.

EXPERIMENTAL PROCEDURE AND RESULTS

Two normal female rats, Nos. 13 and 21, both 61 days old at the beginning of the experiment, were given a series of 8 injections of *T. lewisi* vaccine at 3-day intervals, 0.1 cc of vaccine being inoculated intraperitoneally per 15 grams of body weight. The two females were put in mating cages with male rats beginning 10 days after the start of vaccination, and left for 10 days. Five days after the last injection of the vaccine, the vaccinated animals, which were then pregnant, were tested for immunity by inoculating living *T. lewisi*. One vaccinated rat proved to be completely resistant, no parasites whatsoever being seen in the tail blood during the next 12 days. The other vaccinated rat showed 2 parasites in 50 microscope fields ($\times 430$) one day after the inoculation of the living forms, but was uniformly negative thereafter when examined daily till the twelfth day (see Table 1 for the results of these tests of immunity).

TABLE 1.—*Demonstrating the immunity against T. lewisi of specifically vaccinated mother rats*

Female test rat	No. of trypa- nosomes injected	Parasite counts on designated days after injecting living <i>T. lewisi</i>						
		1	2	4	6	8	10	12
13	8	0	0	0	0	0	0	0
21	8	+	0	0	0	0	0	0
26*	None	—	—	—	—	—	—	—
27	None	+	2+	4+	4+	4+	2+	0

* Two trypanosomes seen in 50 fields of microscope ($\times 430$) on first day only.

Key to parasite counts:

0 = no parasites in 50 microscope fields ($\times 430$).

— = less than 1 trypanosome per field.

— = from 1 to 5 trypanosomes per field.

— = from 5 to 25 trypanosomes per field.

— = about 25 trypanosomes per field.

along with control animals. On the twelfth day after the immunity test, both females delivered litters, No. 13 producing 7 and No. 21 producing 3 young. Three of the young of No. 13 were—before nursing their own mother—transposed with 4 young of normal mother No. 30, which had delivered 8 young on the same day. The 3 young of immune mother No. 21 were permitted to nurse their own mother. Five days after their birth, all the young were inoculated with 70,000 living *T. lewisi*. The observations upon the ensuing infections are presented in Table 2.

The more significant results were obtained with the young of immune mother 13 and of normal mother 30. All of the own-young and 3 of 4 foster-young nursing immune mother 13 remained consistently negative during the 20 days of observation after the living trypanosomes were

TABLE 2.—*Demonstrating the relative resistance against T. lewisi of young which nurse a vaccinated mother rat compared with that of young nursing a normal mother rat*

Young rat no.	Born of mother no.	Nursed by mother no.	Parasite counts on designated days, after injecting live <i>T. lewisi</i>							
			1	2	4	6	8	11	15	20
1	13 (Immune)	13 (Immune)	0	0	0	0	0	0	0	0
2			0	0	0	0	0	0	0	0
3			0	0	0	0	0	0	0	0
4			0	0	0	0	0	0	0	0
5	30 (Normal)		0	0	0	0	0	0	0	0
6			0	0	0	0	0	0	0	0
7			0	0	0	0	0	0	0	0
8			-	0	2-	3-	4-	4-	2-	0
11	(Immune)	30 (Normal)	0	+	4+	4+	4+	4+	dead	
12			0	0	0	+	4+	4+	dead	
13			0	0	0	0	+	4+	4+	4+
14			+	+	4+	4+	4+	3+	2+	0
15	30 (Normal)		+	+	4+	4+	4+	4+	3+	3+
16			+	+	4+	4+	4+	4+	3+	2+
17			+	+	4+	4+	4+	4+	3+	2+
21	21 (Immune)		21 (Immune)	0	0	0	+	0	0	0
22		0		0	0	0	0	0	0	0
23		+		0	0	0	0	0	0	0

Young rats injected when 5 days old with 70,000 *T. lewisi*.

Key to parasite counts: same as Table 1.

injected. The remaining one foster-young, however, contracted a heavy infection. All of the young which nursed the normal mother, in contrast, contracted heavy infections regardless of whether or not their own mothers were immune. Although there appeared some slight delay in the development of the infection in the 3 foster young nursing the normal mother, their infections finally became quite as severe as those of the own young of the normal mother, two of the foster young succumbing to their infections.

All of the 3 young nursing immune mother No. 21 were found to be highly resistant. Two of these manifested the infection, although in each case on but a single day. The third nursing remained negative throughout the 20 days of observation.

DISCUSSION

It is indicated from the results recorded in this paper that mother rats immunized by vaccination with a formolized suspension of *T. lewisi*, transmit to their young the immunity acquired by the vaccination. It is shown that the protective substances responsible for this immunity reach the young largely after birth through the ingestion by the young of the milk of the immune mother, since young born of an immune mother but deprived of her milk by transposition at birth to a normal mother are readily susceptible to severe infections with the homologous parasite.

One detail in the procedure requires further comment. It was pointed

out that five days after the last immunizing injection, the female rats, which were pregnant at that time, were inoculated with living *T. lewisi* to test the production of immunity by vaccination. Although one animal remained completely uninfected, so far as careful tail blood examinations revealed, the other animal showed parasites (2 in 50 fields of the microscope at 430 diameters) for one day following. An objection might be raised, then, that the immunity transmitted to the young resulted from recovery by the mother from an infection with *T. lewisi* rather than from her vaccination with the formalized suspension of this parasite. However, in the opinion of the author, no infection involving the presence of multiplying trypanosomes occurred, the forms found representing merely part of the inoculum of living forms given the preceding day and still in the circulation. Furthermore, it has been shown by Coventry (6) that antibody against *T. lewisi* does not make its appearance in an animal until a significantly heavy infection has been in progress for several days. Parasites were not seen in this work after the first day in the one mother animal which showed them at all. It is concluded, therefore, that the immunity transmitted by these mother rats was developed in them by vaccination and not by recovery from infection.

The character of the antibody transmitted from the mothers to the young probably depends on the character of the antibody in the blood of the mother at parturition and during the nursing period. Taliaferro (7) has shown that rats which have recovered from infection with *T. lewisi* produce two distinct serum antibodies—the one manifesting a trypanocidal action and the other a capacity for inhibiting the reproduction of trypanosomes. He has also demonstrated that the reproduction-inhibiting antibody is developed principally although possibly not exclusively by rats through vaccination. Some small amount of evidence is forthcoming from the present work to indicate that the antibody transmitted to the nurslings from the vaccinated mother rats inhibited the reproduction of the trypanosomes in the nurslings. Of the three young nursing Mother Rat No. 21, for example, two revealed trypanosomes in the blood, one of these (young rat No. 21) as many as six days after the inoculation of living forms. Evidently, the trypanosomes, although present during this six-day period in this animal were not destroyed by the substances obtained through nursing the immune mother, but were merely prevented from increasing in number. Whether the trypanocidal antibody also was transmitted to some of the other nurslings and was responsible for the complete absence of trypanosomes from their blood was not determined.

SUMMARY

Mother rats immunized against *Trypanosoma lewisi* by vaccination with a formalized suspension of this organism transmit their immunity to

their young. The young become immune largely by ingesting the milk of the immune mother. Such immunity as the young may acquire through the placental circulation is largely lost within a few days after birth.

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THE FUNCTION OF RESPIRATORY PIGMENTS OF CERTAIN TURTLE PARASITES*

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Von Brand (1938) discusses in some detail most of the previous work in the field of the respiration of parasitic worms, in an attempt to answer the question whether helminths in the intestines of vertebrates lead an aerobic or anaerobic life. Because of the low oxygen tensions observed in the vertebrate alimentary canal (Tappeiner 1883, Long and Fenger 1917, von Brand and Weise 1932), the worms were considered to have anaerobic metabolism. Anaerobic respiration is known to occur in all of the parasitic worms which have been tested (Wardle 1937a, von Brand 1937b, Stannard, McCoy and Latchford 1938, and many earlier works). Likewise aerobic respiration has been shown to exist. Von Brand (1938) concludes that, "large helminths, like *Ascaris* or *Moniezia*, gain their energy predominantly by fermentation [of glycogen] during their life in the intestine, but that small parasites like sheep nematodes, may lead there a more aerobic life."

Several investigators (Davey 1938, Slater 1928) show that worms in vitro live longer under aerobic than anaerobic conditions. Hoeppli, Feng, and Chu (1938) give a complete reference list of studies of this type. Von Brand (1937b) has shown that *Ascaris* has the ability of resynthesizing glycogen from the breakdown products of anaerobic metabolism when plenty of oxygen is available.

Both hemoglobin and cytochrome have been discovered in nematodes (Keilin 1925), but only cytochrome has been found in cestodes (Friedheim and Baer 1933). Hemoglobin was found to be concentrated in the hypodermis of *Ascaris* (Krüger 1936) where it allegedly aids the organism by removing oxygen from the surrounding medium through the cuticle. *Trichina* larvae were shown to have hemoglobin (Stannard, McCoy, and Latchford 1938) which probably aids somehow in their respiration. Von Brand (1937a) found hemoglobin in another larval nematode.

Aducco (1889) carried out several experiments on the red pigment in the cuticle and body fluids of *Diectophyme renale* (Goeze 1782). The pigment was found to be similar to vertebrate hemoglobin, but much more resistant to change by physical and chemical agents. The pigment from *D. renale* could not be completely reduced with a vacuum pump, but

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with the aid of chemical reducing agents, complete reduction of it was accomplished.

Wardle (1937a and b), von Brand (1933, 1937b, 1938), and others have shown that the oxidizable substrate in parasites is glycogen and that when energy is produced, the glycogen is fermented to CO_2 and lower fatty acids. Krüger (1936) has shown the acids to be valeric and capronic in the case of *Ascaris*. In the presence of oxygen, aerobic oxidation also takes place.

Very little work has been done on the oxygen-obtaining mechanisms of parasites. Wells (1931) has demonstrated that a hookworm sucks blood from the host which would permit it to obtain oxygen from erythrocytes. Von Brand (1938) points out that 0.2 mg of O_2 would be available daily to each worm from this source.

As has already been mentioned, respiratory pigments are known to occur in parasites, but the mechanics of their action have not been determined, hence an attempt to discover the role of hemoglobin in the respiration of turtle parasites was undertaken.

MATERIALS AND METHODS

Two trematodes (*Telorchis robustus*, *Allasostoma magnum*) and three nematodes (*Camollanus trispinosus*, *Falcaustra affine*, and *Crucia testudinis*) were tested for hemoglobin. A suitable number of worms, equivalent to about 1 cc in volume, if that many were available, were removed from the host, washed from 1 hour to 48 hours in salt solution at 4° C, placed in a 15 cc centrifuge tube and frozen in brine. Then 0.5 cc of phosphate buffer (pH 6.8) and 0.5 cc of distilled water were added to the tube, and the contents repeatedly frozen and thawed, usually five or six times, until there was no color remaining in the worms. The solutions were centrifuged and the clear supernatant fluid was removed by a pipette to a test tube. If the worms contained hemoglobin, then this was the hemoglobin solution used in the experiments. The residue from some extractions of *C. trispinosus* and *A. magnum* were re-extracted with 1.5 cc of weak ammonia solution containing a small amount of sodium hydrosulfite. This was centrifuged and the supernatant fluid was tested for cytochrome.

Spectroscopic examinations were conducted in three ways: (1) the solution was spectrographed and the plate analyzed by a photoelectric densitometer; an iron arc spectrum was photographed on the same plate for reference (Dr. L. G. Bonner assisted in this phase of the work); (2) the solutions were examined by eye with a comparometer spectroscope; (3) a Zeiss microspectroscopic eye piece was used with an ordinary compound microscope to detect hemoglobin in individual worms.

An oxygen dissociation curve of a single sample of the hemoglobin

from *C. trispinosus* was determined with the aid of Dr. F. G. Hall and his micro-method for dilute hemoglobin solutions (Hall 1934, 1935).

The ability of *C. trispinosus* to reduce its own hemoglobin was determined in two ways: (1) several worms were removed from a turtle, washed in salt solution, placed in a fine capillary tube which was filled with saline and closed with paraffin at both ends. The worm was examined with a microspectroscope. Later the tube was broken, the worms removed and examined in the same manner in a small drop of saline which was exposed to the atmosphere; (2) a spectro-comparator such as described by Hall (1935) was used in place of the ocular of a compound microscope. On the mechanical stage of the microscope was fastened a fine glass tube containing a worm for examination, which was connected with a reservoir of saline so that fluid could be passed through the tube at any desired speed; the spectral bands of the worm could thus be matched by absorption bands of oxi- and reduced-hemoglobin solutions of known proportions.

RESULTS

Reddish extractions were obtained from *Telorchis robustus*, *Allasostoma magnum*, and *Camallanus trispinosus*. When these solutions were examined spectroscopically, they were found to have two absorption bands, one at about 5,750 Ångström units and the other at about 5,400. These bands disappeared when the solutions were reduced with sodium hydrosulfite, and were replaced by a single broad band in a region between the two former bands (Figs. 1 and 2). Hemoglobin solutions



FIG. 1. Spectrographs of hemoglobin from *Telorchis robustus*. 1. Reduced hemoglobin. 2. Partially reduced hemoglobin. 3. Oxy-hemoglobin.

behave in this manner, hence it is assumed that hemoglobin lends the reddish color to these solutions. *Falcaustra affine* and *Cruzia testudinis* were not found to have hemoglobin.

The second extracts from *A. magnum* and *C. trispinosus* showed when reduced a faint absorption band at 5,500 Ångström units which was probably the strong band of cytochrome C.

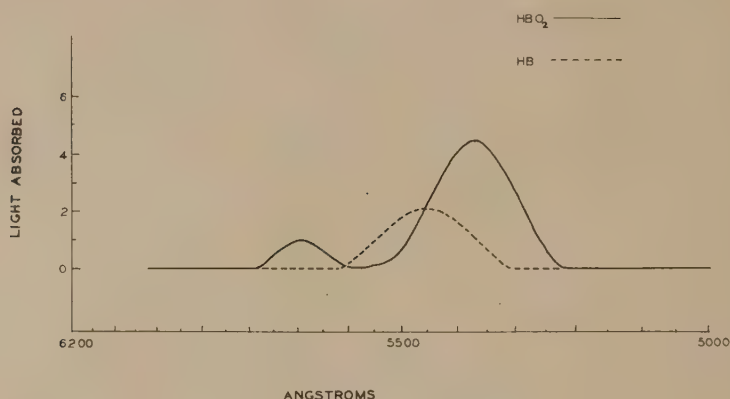


FIG. 2. The relative shape and position of the absorption bands of *Telorchis robustus* hemoglobin. Amount of light is given in arbitrary units. The position of the bands is not accurate to more than 100 Ångström units.

The oxygen dissociation curve obtained for the hemoglobin of *C. trispinosus* is given in Fig. 3, along with the curve of the host hemoglobin as determined under similar conditions by Wilson (1938). The curve for the parasite pigment is far to the left of that of the host.

When in anaerobic surroundings, the hemoglobin of *C. trispinosus* is in a reduced state, but when the worm is in contact with oxygen, the hemoglobin is in a partially oxidized condition, the extent of oxidation depending upon the partial pressure of the oxygen.

T. robustus, *A. magnum*, and *C. trispinosus* cling to the intestinal wall by their mouths, where they cause inflammation. When these worms are removed from their points of attachment, their former positions are at times indicated by small red "pimples." *F. affine* and *C. testudinis* live free in the lumen of the gut.

DISCUSSION

That a parasite has hemoglobin not identical with that of its host is shown by the difference in the two oxygen dissociation curves (Fig. 3). Whether or not the raw materials for the manufacture of hemoglobin by the parasite are obtained by digesting host hemoglobin and absorbing the decomposition products, is not known, nor is it important in the consideration of the oxygen-obtaining mechanism. Telorchids usually live just posterior to the bile ducts, so perhaps they obtain their essential substances for synthesis from the bile.

Turtle parasites that contain hemoglobin are all in close contact with the blood stream of the host. The dissociation curve of the host pigment is far to the right of that of at least one of the worms, which signifies that

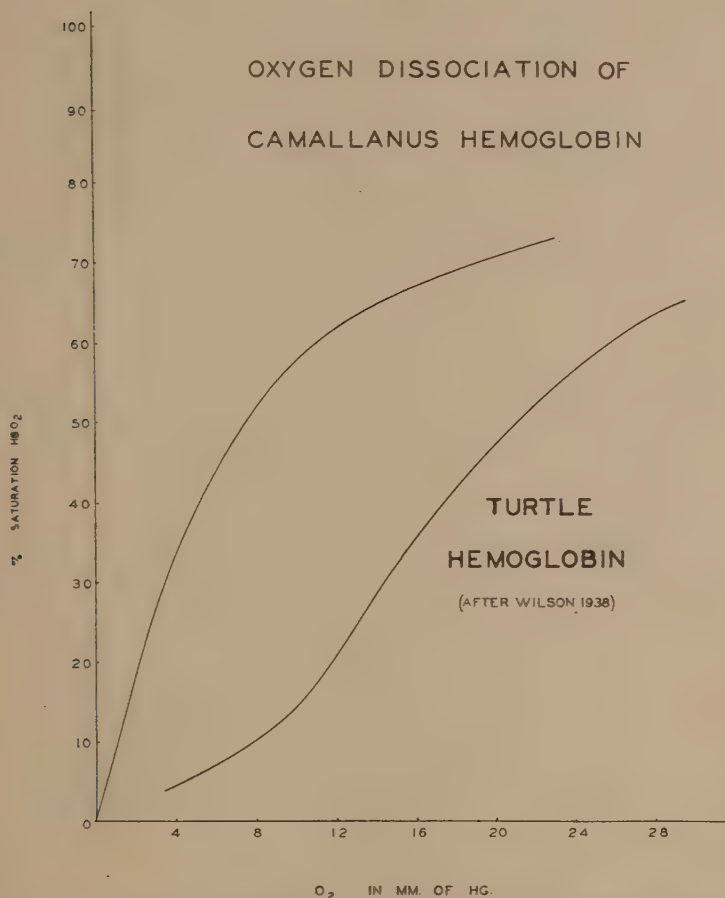


FIG. 3. Oxygen dissociation curves. Hemoglobin from *Camallanus trispinosus* and its host *Pseudemys troostii* tested under similar conditions.

the hemoglobin of the parasite can take up oxygen at very low pressures or even from the venous or reduced blood of its host. The work of Aducco (1889) on the red pigment of *D. renale* showed that its affinity for oxygen was much greater than that of vertebrate hemoglobin. In fact the properties of the red pigment of *D. renale* agree so closely with those of the worm hemoglobins discussed in this paper, that there is little doubt that the pigment described by Aducco is in reality hemoglobin. *C. trispinosus* can reduce its own hemoglobin, probably with the aid of cytochrome which acts as an oxygen transport mechanism between the

hemoglobin and the oxidizable substrate (glycogen ?, von Brand 1938). These facts show that it is possible for oxygen to be taken from the host blood by the hemoglobin of *C. trispinosus*, and that this stolen oxygen can be used in the metabolic processes of the worm. That this occurs is supported by the fact that those worms which live free in the lumen have not been found to contain hemoglobin. It can not be said that this mechanism has actually been demonstrated for *C. trispinosus*. It has been shown to be physically possible even probable, but until some means of observing the phenomenon in its entirety is devised, final judgment must be withheld. A mammalian embryo has a similar mechanism for obtaining its oxygen from its mother's blood (Evans 1936).

CONCLUSIONS

1. Hemoglobin is present in *Telorchis robustus*, *Allassostoma magnum* and *Camallanus trispinosus*.
2. Cytochrome is present in *A. magnum* and *C. trispinosus*.
3. Hemoglobin is probably not present in *Falcaustra affine* or *Cruzia testudinis*.
4. Host and parasite hemoglobin are not identical.
5. The respiratory pigments of these parasites probably aid them in obtaining oxygen from the host blood.

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RESEARCH NOTES

INCIDENCE OF *ENDAMOEBA HISTOLYTICA* AND INTESTINAL NEMATODES IN A GEORGIA STATE INSTITUTION

Through the kindness of Dr. John W. Oden, Superintendent of the State Hospital at Milledgeville, Georgia, a parasite survey was made during 1938 on a number of patients at the institution. One ward of 72 white adult females was examined in June for intestinal parasites, and again in November when the ward contained 70 patients. Mentally the patients examined fell into various classifications; physically all were considered to be healthy adults and had been in residence at the institution from several months to many years. An additional ward of 88 white females was examined in October for pinworm infection only. No information concerning the mental or physical status or length of institutional residence of these patients was obtained.

The parasite examinations were made as follows: For *Endamoeba histolytica* and *Strongyloides stercoralis* saline smear and iodine smear examinations from 3 stools per patient; for worms except *Strongyloides stercoralis* and *Enterobius vermicularis* brine flotation examinations from 2 stools per patient; for *Enterobius vermicularis* 3 anal swabs per patient.

Concurrent with the parasite examinations Stoll egg counts on 3 stools per patient were made to determine the intensity of certain of the worm infections. These determinations were calculated from the data of Leathers and Keller (1935, New Orleans Med. and Surg. J. 87: 425-435) and Keller and Leathers (1936, Am. J. Hyg. 23: 216-230).

The results of the parasite examinations are summarized in the following table. No tapeworm infections were encountered.

TABLE 1.—Record of positive diagnoses for intestinal parasites

Date	Patients examined	<i>Endamoeba histolytica</i>	<i>Ascaris lumbricoides</i>	<i>Necator americanus</i>	<i>Trichuris trichiura</i>	<i>Strongyloides stercoralis</i>	<i>Enterobius vermicularis</i>
1938	No.	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
June	72	40	14	33	65	6	53
November . .	70	44	9	30	61	6	69
October . . .	88	*	*	*	*	*	27

* No data obtained on these parasites.

The parasite findings given above have been analyzed as follows:

Endamoeba histolytica. The importance of the high incidence of this parasite appears to be minimized by the fact that there was no diarrhea or dysentery among the patients examined and for the most part all stools observed were normal, formed stools. In patients of the type examined only such objective symptoms as diarrhea or dysentery could be used as an index of the pathogenicity of the parasite and the complete absence of such symptoms suggests that the organism was one of low virulence or that a resistance had been acquired due to long-continued exposure implicit in the insanitary habits of this type of patient.

The analysis of the nematode findings was based on the egg counts made during the June examinations. A similar analysis made in November revealed no appreciable differences and the data are therefore not cited.

Ascaris lumbricoides. The egg counts of the 10 positives for this parasite indicated that all infections were light.

Necator americanus. In only one case did the egg counts for this parasite indicate a heavy infection. Two cases were considered moderate infections and the remainder light.

Trichuris trichiura. With this parasite 5 cases out of 47 were regarded as heavy or very heavy infections. Seventeen were considered moderate and the remainder light.

No estimation of intensity of infection with *Strongyloides stercoralis* was possible. Nor could intensity of infection with *Enterobius vermicularis* be gauged since the number of ova on a swab is no index of the number of worms in the intestine.

The foregoing analysis of the findings for both *E. histolytica* and for the helminth parasites indicate that the parasite burden of the patients was, on the whole, light although the incidence of parasitism was high. In an institution of this type a high incidence of intestinal parasites is not necessarily surprising in view of the fact that the communal domiciling of mentally deranged persons provides very favorable conditions for the spread of parasitic infections.—LUCY V. REARDON, *Associate Protozoologist, National Institute of Health, U. S. Public Health Service, Washington, D. C.*

INTESTINAL PROTOZOA IN 106 PARASITOLOGY STUDENTS

In a survey of a class of 45 parasitology students at Dartmouth College, Connell and French (1939, J. Am. Med. Assn. 113: 649-652) reported 3 with *Entamoeba histolytica* and 10 with one or more species of amoeba.

Similarly, as a regular feature of the work in the course in protozoan parasitology at the University of Pennsylvania, each student is given a fecal examination for intestinal protozoa. In Table 1 are summarized the results of several years' efforts. One normally passed stool was obtained from each student and the specimens averaged 17.1 hours old at the time of examination. The diagnoses were made from saline, iodine and permanent preparations of each sample.

TABLE 1.—Percentage of protozoa in 106 students

Positive for protozoa	Negative for protozoa	<i>Entamoeba histolytica</i>	<i>Entamoeba coli</i>	<i>Endolimax nana</i>	<i>Dientamoeba fragilis</i>	<i>Giardia lamblia</i>	Multiple infections	<i>Blastocystis hominis</i>
34	66	8	8	13	3	11	8	69

The absence of such forms as *Iodamoeba*, *Trichomonas*, etc is probably due to the relatively small number examined. Of these 106 students, 59 were residents of Philadelphia, while Pennsylvania, New Jersey and Delaware accounted for 94. Of the 8 harboring *E. histolytica*, 5 were Philadelphians.—ROBERT M. STABLER, *Department of Zoology, University of Pennsylvania.*

THE OCCURRENCE OF LARVAE OF THE STABLE FLY, *MUSCINA STABULANS* (ZETT.) IN LIVING NYMPHS OF THE GRASS-HOPPER, *XANTHIPPIUS CORALLIPES* PANTHERINUS (SC.)

During the course of some studies of the immature stages of certain grasshoppers, two second-instar nymphs of *Xanthippus corallipes pantherinus* (Sc.) were observed in the field near Dallas, Texas, on November 9, 1939 to be unusually inactive and upon immediate dissection of these living specimens five larvae of *Muscina stabulans* (Zett.) were obtained from each nymph. Five other nymphs collected at the same time were confined in screen-tight laboratory cages and upon dissection five days later, three were similarly infected.

Collections made in the same locality on April 20, 1940 revealed that three of four fourth-instar nymphs and five of seven fifth-instar nymphs were infected. Likewise three days later similar collections showed two of seven fourth-instar nymphs and six of eight fifth-instar nymphs to be infected. In all instances the infected individuals exhibited the same retarded activity. Collections of seven adults on May 12 and eight adults on May 21 failed to reveal any larvae.

Mr. D. G. Hall has kindly identified the species, from adults reared from the larvae, as *Muscina stabulans* (Zett.).

When dissected out, the larvae are active and inclined to crawl several inches or feet, whereupon the older individuals pupate, although in the laboratory cages three actually penetrated weakened living nymphs of two other grasshoppers, *Encyptolophus sordidus costalis* (Sc.) and *Chortophaga viridifasciata* (DeGeer). The number of maggots in a nymph varied from one to eighteen, with a mean of eight.

In laboratory cages pupation occurred (90 specimens) from April 29 to May 6. The pupae were usually embedded in the soil from one-half to one and one-half inches, although nine were found on the surface of the soil under dead vegetation. The adults emerged from May 7 to May 12.

Many reports of the rearing of this fly from various orders of insects have been recorded in the literature, although its actual status as a parasite is often disputed, since no records are to be found which record a complete parasitic part of the life cycle. The fact that the larvae can be found in living, moving nymphs, however, would seem to give additional support to the view that the fly is, under these conditions, actually parasitic, possibly entering the host as a larva, and may represent a transitional stage in the evolution of parasitism.—HERBERT KNUTSON, *Biology Department, Southern Methodist University, Dallas, Texas.*

TAMERLANEA BRAGAI, A PARASITE OF PIGEONS IN PUERTO RICO

During October, 1938, one of us (J. M.) recovered a trematode from a pigeon which came from a farm at Adjuntas, central Puerto Rico. Nineteen of twenty birds in this locality harbored the parasite. Considerable difficulty was at first experienced in the identification of the fluke. An examination of the figure of *Tamerlanea bragai* Santos (Reis and Nobrega, 1936, Doenças das Aves, Sao Paulo, Fig. 228, p. 313) convinced us we were dealing with this species, an opinion confirmed by Dr. E. W. Price and Mr. A. McIntosh of the U. S. Bureau of Animal Industry. Specimens kindly forwarded by Professor Santos from Brazil were received in poor condition and could not be satisfactorily stained to permit comparison.

The parasite was restricted to the kidney and urethra. The number of flukes recovered varied from two to twenty. Pigeons kept in cages for several months appeared unaffected by their infections. An inflamed urethra was noted in one pigeon at autopsy. According to Dr. Krakower of the School of Tropical Medicine: "The effects of the parasite upon the kidneys are chiefly mechanical, that is, by dilation of the pelvis and collecting tubules, without, however, interfering with the renal function. The dilation is sufficient to allow this. The muscular hypertrophy of the walls is compensatory, probably to expel the parasite from the lumen. The slight excess of round cell infiltration indicates that the irritation set up by the parasite is minimal."

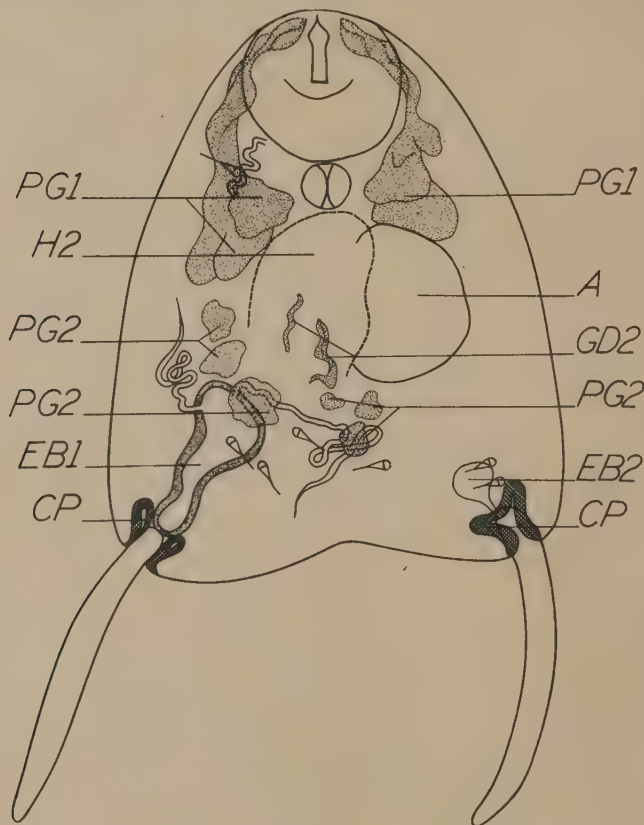
Though first found in the interior of the Island, the recovery of adult flukes from autopsied pigeons at Rio Piedras, and of the typical small brown ova from the excreta of the pigeons in several localities between San Juan and Rio Piedras, suggest that the species is widely distributed in Puerto Rico. This marks the second time a trematode known from South America has been found in Puerto Rico without the interpolation of any known intermediate distributional records. The first instance was that of *Platynosoma concinnum*, common locally in the livers of cats.

The ova of *T. bragai*, when passed, contain fully developed miracidia which fail to hatch in water or charcoal cultures. This seems to indicate ingestion by the molluscan host.—JOSÉ F. MALDONADO, *Insular Agricultural Experiment Station, Rio Piedras, P.R.* AND W. A. HOFFMAN, *Department of Medical Zoology, School of Tropical Medicine, San Juan, P.R.*

PARTIAL TWINNING IN A STYLET CERCARIA

In the course of a study of development of the cercaria of *Alloglossidium corti* an abnormal nearly mature cercaria was encountered in material obtained by dissection of the sporocyst.

As shown in the figure (composite drawing from both living and stained speci-

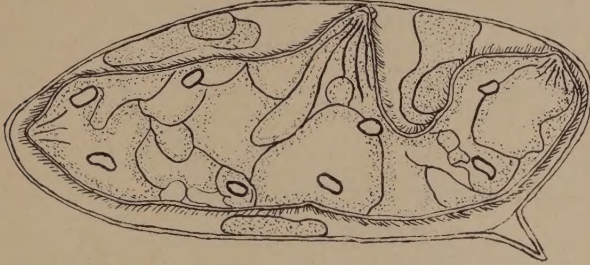


men), the posterior part of the cercaria is almost completely doubled. Both tails were actively moving and appeared normal, although the movement of the cercaria as a whole was rather sluggish when compared with normal cercariae of a similar stage of development. Caudal pockets (cp) are distinct around both tails. There is a partially developed second head region (h 2) near the acetabulum (a). The apparently normal head region has stylet, oral sucker, pharynx and ducts of penetration glands; the penetration glands (pg 1) have indistinct boundaries; intestinal caeca are not visible. Penetration glands (pg 2) and their ducts (gd 2) associated with the second head region (h 2) were observed. A second stylet was noted by one observer, but was not seen again in the living specimen, or after staining. Of the two excretory bladders one (eb 1), large and thick-walled, appears normal and receives two collecting tubes; the other (eb 2) is small, with indistinct wall and no visible collecting tubes. Several flame cells were visible but the flame cell pattern could not be determined.

It is entirely possible that such an abnormal cercaria would never emerge from the snail.—KATHLEEN L. HUSSEY, *Department of Zoology, University of Michigan.*

MIRACIDIAL TWINNING IN *SCHISTOSOMA MANSONI*

While making examinations of the excreta of a patient infected with *Schistosoma mansoni*, an egg of normal size was found containing a pair of miracidia (See Fig.). The miracidia were fused anteriorly for about one third of their



length and in each of them a normal set of four active flame cells was observed. The head was located in the middle portion of the egg, the posterior end of the miracidia situated at each extremity. The paired organisms were quite active.

Miracidial twinning has been reported in excreta from a monkey (Hoffman and Janer, 1936, *Proc. Helm. Soc. Wash.* 3: 62). At the time it was believed that the host may have in some way caused this abnormality. The present finding indicates the phenomenon may occur in man, as well.

Penetration of the molluscan host by these miracidia, were they more abundant, might give rise to some interesting genetic conditions.—JOSÉ L. JANER, *Department of Medical Zoology, School of Tropical Medicine, San Juan, P.R.*

A STOMACH TUBE FOR MICE

Male catheters are in general use as stomach tubes for rats, guinea pigs, and rabbits. These tubes, however, are not of sufficiently small diameter to be used for mice. Glass tubes have proved unsatisfactory in the hands of the author due to lack of flexibility of the glass. A small stomach tube has therefore been improvised from cellophane and so far has proved successful in use on mice.

Different grades of cellophane are available commercially, but the author has used only one type, the cellophane ordinarily used for wrapping purposes. This is about the same weight as that used on cigarette packages. The cellophane is cut into trapezoidal strips (Fig. 3) and, after one side of the lower right hand corner has been dampened, the edge is rolled (Fig. 2) between the thumb and index finger. The angle at which the cellophane is rolled (Fig. 2, 115°) in relation to the longitudinal axis of the paper will determine the length of the resulting tube and consequently the number of layers composing the wall. After completion of the rolling process, the distal end of the paper is cemented and since the tube is rolled tightly, this is sufficient to hold it together and keep it from telescoping.

From the experience of the author, a very useful tube is made by starting at an angle of approximately 115° on a cellophane strip of the size shown in Fig. 3. After the cellophane has been rolled to form a tube approximately $\frac{3}{4}$ inches in length, the angle of rolling is increased slightly each time the tube is turned so that a tube results (Fig. 1) which measures about $3\frac{1}{4}$ inches long. One quarter inch is then cut from the small end and $\frac{1}{2}$ inch from the large end. The resulting tube has a diameter at the larger end of approximately 2 mm and a diameter at the smaller end of slightly less than 1 mm. The edge of the small end should be smoothed with fine sand paper. A hypodermic needle of the proper size, with the point filed off, is inserted into the large end of the tube when in operation.

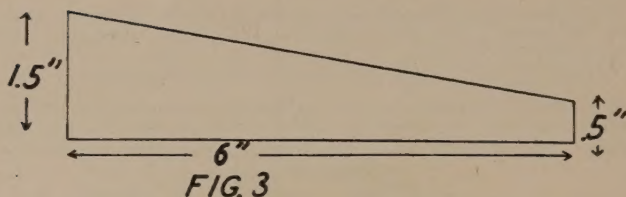


FIG. 3

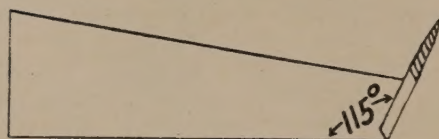


FIG. 2

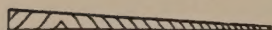


FIG. 1

Mice are difficult to restrain manually so a restraining bag with a zipper, stocks, or some other device should be used for holding the animal. It is necessary to press on the extreme base of the mandible on either side in order to keep the mouse's jaws apart and hence avoid having the tube bitten through. This pressure is most easily applied by the fingernail of the index finger while the thumb holds the back of the animal's head. Care should be taken to avoid pinching the trachea. Some practice is necessary in order to apply pressure correctly. In an alternative method a small glass tube is inserted into the mouth and provides a passageway for the stomach tube thus protecting it from the incisors.

The stomach tube, previously coated with a lubricant, should be inserted by following mid-dorsally down the roof of the mouth, exercising care to avoid contact with the molars and tracheal orifice. The insertion of the needle into the stomach tube and injection should be accomplished as quickly as possible since mice seem unable to breathe while the stomach tube is in situ.

The author is using this tube for the administration of anthelmintics to mice but it should serve equally well for infectious material, vitamin concentrates, etc.—G. M. SPURLOCK, *Division of Entomology and Parasitology, University of California, Davis, California.*

REVIEW

THE INVERTEBRATES: Protozoa through Ctenophora. By Libbie Henrietta Hyman. xii + 726 pp. 221 illustrations. McGraw-Hill Book Company, Inc., New York. 1940. \$7.00.

This book is part I of volume I of a projected three-volume reference work on the invertebrates. It opens with two introductory chapters, the first (21 pp) dealing with protoplasm, the cell and the organism; and the second (18 pp) with classification. Chapters III to VIII deal respectively with Phylum Protozoa (90 pp); Phylum Mesozoa (15 pp); Introduction to the Lower Metazoa (35 pp); Phylum Porifera (80 pp); Phylum Cnidaria (296 pp) and Phylum Ctenophora (35 pp). Each chapter includes at its end a fairly extensive bibliography and the volume closes with an index of 30 pages.

Chapter I begins with a consideration of the physical, chemical and biological properties of protoplasm and continues with a discussion of the cell and its parts, the cell theory and cell division. The organism is then considered with attention to form, symmetry, metamerism, polarity, etc.

In Chapter II, on classification, the subject is treated historically and then a system is adopted which recognizes 22 phyla. Subkingdom I consists of Phylum Protozoa. Subkingdom II, the Metazoa, comprises three branches: A, Mesozoa (Phylum Mesozoa); B, Parazoa (Phylum Porifera); and C, Eumetazoa. The latter consists of Grade I, Radiata, composed of the phyla Cnidaria and Ctenophora, and Grade II, Bilateria. This grade consists of three groups: a, Acoelomata (Phyla Platyhelminthes and Rhynchocoela or Nemertinea); b, Pseudocoelomata (Phyla Aschelminthes—rotifers, nematodes, etc.—and Entoprocta); and c, Eucoelomata. The Eucoelomata are further divided into 1) The Schizocoela, in which are included the Phyla Bryozoa or Polyzoa, Phoronida, Brachyopoda, Mollusca, Sipunculoidea, Priapulioidea, Echiuroidea, Annelida and Arthropoda; and 2) The Enterocoela which contains the Phyla Chaetognatha, Echinodermata, Hemichorda and Chordata.

Chapter III on Protozoa begins with a section on the characters of the phylum in which it is stated that they are acellular rather than unicellular, followed by an outline of classification, and a discussion of the general morphology and physiology of the group. This valuable discussion (34 pp) includes the topics of form and size, general structure of the body, the nucleus, encasements and shells, kinetic elements, locomotor organelles, nutrition, respiration and the contractile vacuole, neuromotor system and sense perception, behavior, encystment, nuclear phenomena of division, asexual reproduction and the division of the cytosome, sexual reproduction, regeneration, genetics, and ecology. Under ecology the terms symbiosis, commensalism and parasitism are defined and discussed.

In the classification of the Protozoa the phylum is first divided into the Subphyla Plasmodroma and Ciliophora. The Plasmodroma include the Classes Flagellata or Mastigophora, the Rhizopoda or Sarcodina, and the Sporozoa. The Flagellata consist of the Orders Chrysomonadina, Cryptomonadina, Dinoflagellata, Chloromonadina, Euglenoidina, Phytomonadina or Volvocales, Protomonadina, Polymastigina, Hypermastigina and Rhizomastigina or Pantostomatida. The Class Rhizopoda includes the Orders Amoebozoa or Lobosa, Foraminifera, Heliozoa and Radiolaria. The Mycetozoa are relegated to the Fungi and hence omitted. The shelled Proto-myxa are placed in the Foraminifera. The Sporozoa are divided into four Subclasses: 1) The Telosporidia, consisting of the Orders Gregarinida, Coccidia and Haemosporidia; 2) The Cnidosporidia, consisting of the Orders Myxosporidia, Actinomyxidida and Microsporidia; 3) Sarcosporidia, which includes the Orders Sarcosporidia and Glabrida; and 4) Haplosporidia, which is not divided into orders.

In the Subphylum Ciliophora, two Classes, Ciliata and Suctorina are recognized. The Ciliata are divided into the Subclasses Protociliata and the Euciliata. The latter is divided into four Orders. The Order Holotricha contains the Suborders Gymnostomata, Trichostomata, Hymenostomata, Astomata and Apostomea. The Order

Spirotricha includes the Suborders Heterotricha, Oligotricha and Hypotricha. The Peritricha and Chonotricha are recognized as separate orders.

Each major taxonomic section opens with a definition of the group, a statement of its general characters and a discussion of its morphology and physiology; then follows an account of the morphology, life history, etc of members of the successive orders. The chapter closes with a section on general and phylogenetic considerations and a bibliography of nearly 25 pages set solid.

As with the other chapters, the one on Protozoa is extensively illustrated. There are 60 figures in this chapter but most of these are full-page groups of drawings so that a total of several hundred drawings is represented. Most of the drawings have been redrawn from a variety of sources but many are original having been drawn from actual specimens.

Typographical errors have been reduced to a minimum, but there are occasional statements with which one might disagree. For example, on page 13 it is stated that the mitotic spindle is seen only in fixed preparations: "The spindle and astral fibers apparently do not exist as such in life but may be fluid channels in the cytoplasm." There are a good many statements to the contrary in the literature and one might refer to the work of Cleveland and his associates who state that the spindle fibers can be plainly seen in living specimens of *Barbulanympha*. On page 25 it is stated that "Das Thierreich" was begun in 1896, whereas one section of this work appeared as early as 1873. However, considering the amount of ground covered, the information is remarkably well-digested and up-to-date.

As is to be expected in a general reference work such as this, the parasites are not given disproportionate attention, although by no means neglected. Those groups consisting entirely of parasites such as the Hypermastigina, Astomata and Sporozoa are presented with as much detail as other groups. In sections containing few or many parasites, these are given attention proportionate to their numbers and importance. For example, in the section devoted to the Order Protomonadina, the discussion of the haemoflagellates, because of their importance, makes up the bulk of the section.

Only one other group covered by this volume is of special interest to parasitologists and that is the Phylum Mesozoa. To the reviewer the chapter on this enigmatic group seems adequate and modern.—D. H. WENRICH.

Dates for mailing of numbers of volume 26 (1940):

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No. 2, April 10.

No. 3, June 10.

No. 4, August 8

No. 5, October 9

No. 6 (with December Supplement), December 11.